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Further Studies of Antigenic Structure of *Pasteurella pestis* in Gels.* (22260)

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The characterization of the antigens of *Pasteurella pestis* was begun by Schütze(1), who studied them using his serologic absorption and precipitin tests and identified a somatic thermostable and an envelope thermolabile antigen. The double diffusion method of Oudin(2) has provided another means of identifying additional antigens of *P. pestis*—10 by Ranson *et al.*(3), 8 by Burrows(4), 7 by Bhagavan *et al.*(5) and by us. Applying the Ouchterlony method(6) to living culture (7) revealed only 4 precipitation zones, but it was suspected that very small amounts of antigen might have remained undetected due to an excess of antibody in the antiserum used. It was learned that additional antigens do become evident if the culture is first concentrated by fractionation. Using the concentrated extract from *P. pestis* cells or the maximum growth of a shake culture supernatant as antigen in the Oudin test(2) has revealed 3 additional antigens.

Materials and methods. Strains. Those tested were one virulent strain, 195/P, isolated in a case of human plague in India, and 4 avirulent strains—E. V. 76(8,9), TRU(10) from the Java strain Tjiwidej R, TJS(11) and B1456-4(12) isolated by single colony picking from virulent strain B1456. The *P. pseudotuberculosis* strain was the avirulent strain 32, received from Dr. E. Thal, State Veterinary Medical Institute, Stockholm, Sweden. *Antisera.* The highly potent antiavirulent *P. pestis* gamma globulin was prepared commercially† from serum of rabbits hyperimmunized with living strain A1122. The *P. pseudotuberculosis* antiserum was prepared in this laboratory by hyperimmunizing rabbits with strain 32. *Agar.* A 1.6% Difco bacto-agar was prepared in normal saline and

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† By E. R. Squibb & Sons or Lederle Laboratories.

was sterilized by autoclaving at 15 lb pressure for 15 minutes. *Preparation of antigens.* Each strain was cultured in a casein hydrolysate mineral glucose medium(13) in a 1 liter wide-mouth Erlenmeyer flask containing 250 ml of the medium. The flask was fixed on a shaking machine kept in a 37°C incubation room. The culture was grown for 7 days and was divided according to the demands of the test.

The 5 methods of treating the cultures were (1) adding formalin to make a 1% solution and allowing the mixture to stand at room temperature for 48 hours, (2) adding toluene to make a 1% solution and allowing it to stand at room temperature for 48 hours, (3) placing it in a boiling water bath for 1 hour, (4) treating it with formalin as in (1) and then placing it in a boiling water bath for 1 hour and (5) treating it with toluene as in (2) and then placing it in a boiling water bath for 1 hour. Strains 195/P and B1456-4 were treated by all five methods, and the remaining 3 strains were treated only by the first two methods. The culture was then centrifuged in an angle rotor of a refrigerated centrifuge at 15000 rpm until all of the organisms had been deposited (approx. 30 minutes). The supernatant fluid was carefully removed and was ready for immediate use. *Preparation of tubes* (Oudin method). 1.6% agar in saline with 0.01% merthiolate was melted and maintained at 50°C. 0.7 ml of a 1:2 dilution of anti-A1122 gamma globulin was mixed with 0.3 ml of the melted agar. 0.5 ml of the mixture was delivered into a sterilized tube (8 mm x 80 mm). After the agar hardened, a second layer of 1 ml of 0.8% agar was carefully delivered over the bottom layer with a capillary pipette and allowed to become solid. 0.5 ml of the antigen preparation was delivered onto the top of the second agar layer, and a drop of toluene was added as preservative. The tubes were sealed with rubber stoppers and placed in the refrigerator overnight. The tubes were transferred to a 37°C incubator. Readings were made at the 3rd, 7th and 15th days.

Results. The visible precipitation zones appeared after 3 days of incubation when the antigens and antibodies had diffused into the plain agar layer. The number of zones depended on the antigen preparation used (Table I).

TABLE I. Number of Antigenic Components of *P. pestis* and *P. pseudotuberculosis* Revealed by the Oudin Test.

Antigen*	Treatment of antigens	No. of precipitation bands — Antiserum — *P. pestis* A1122 gamma globulin	No. of precipitation bands — Antiserum — *P. pseudotuberculosis* 32
195/P	Formalin	7	5
B1456-4		7	5
TJS		7	5
E.V. 76		7	5
TRU		7	5
195/P	Toluene	7	5
B1456-4		7	5
TJS		7	5
E.V. 76		7	5
TRU		7	5
195/P	Boiling	3	2
B1456-4		3	2
195/P	Toluene and	3	2
B1456-4	boiling	3	2
195/P	Formalin and	1	1
B1456-4	boiling	1	1

* 195/P is a virulent strain and the remaining 4 are avirulent.

With *P. pestis* antiserum, the chemically killed virulent and avirulent strains all contained 7 soluble antigens; 4 zones were intense and appeared earlier than the remaining 3. In the Oudin test 3 zones formed in the boiled preparation, 3 in the toluene-killed and boiled preparation and 1 in the formalin-killed and boiled preparation.

When *P. pseudotuberculosis* antiserum was used with the *P. pestis* strains, 5 zones appeared with the formalin or toluene treated, 2 with the boiled and the toluene-treated and boiled, and only 1 with the formalin-treated and boiled.

All of the thermostable antigens of *P. pestis* except the haptenized Fraction I were common to *P. pseudotuberculosis.*

Discussion. When the naturally occurring bacterial cells of *P. pestis* and *P. pseudotuberculosis* strains and their fractions were tested with anti-*P. pestis* gamma globulin by means of a modified Ouchterlony plate technic, 2

antigen-antibody precipitation reactions were common to both organisms, but Fraction I (envelope antigen) and II (toxin) reactions were specific for *P. pestis*(7). The very slight amount of Fraction I in strain 14 and TRU was not revealed, and this was to be expected because the antigens could not reach equivalence concentration.

The Oudin test revealed more antigenic components: All virulent and avirulent strains formed 7 precipitation zones, 5 of which were also common to *P. pseudotuberculosis*. Four of the corresponding antigens in the anti-*P. pestis* serum tube and 2 in the anti-*P. pseudotuberculosis* serum tube were distinctly precipitated. These 4 should therefore be considered major quantitatively. The others more faintly precipitated always appeared slowly.

Of the 7 antigens, 4 were thermolabile and 2, thermostable. The remaining antigen was the haptenized Fraction I. Of the antigens common also to *P. pseudotuberculosis,* 3 were thermolabile and 2, thermostable.

Schütze(1) pointed out that steaming the bacterial suspension in saline for a short time (¼ hour) haptenized the envelope antigen, but that it still precipitated in his precipitin test with its corresponding antibody. Steaming for 1 hour destroys it for *in vitro* reactions. But even after boiling the casein hydrolysate mineral glucose grown culture for 1 hour, the Fraction I (envelope antigen) antigen-antibody reaction zone still was sharply defined in the Oudin test. Boiling for 1 hour haptenizes Fraction I, but it will still form a precipitate with its corresponding antibody. This has been learned by boiling the highly purified Fraction I in different concentrations, using it as an external reactant.

Also in the complement-fixation test(14) using 4 combining units of the anti-Fraction I serum to react with the supernatant of the boiled and of the toluene-killed and boiled suspension, the reaction has been positive. The complement-fixation test was negative when the formalin-killed and boiled preparation was used.

The fact that 3 zones appeared in the boiled and in the toluene-killed and boiled antigens and that only 1 faint zone appeared in the formalin-killed and boiled antigen indicates that formalin affects the antigens and renders them thermolabile.

The technic of double diffusion in gels is of apparent value for comparative studies of heterologous, but closely related, organisms (7). More than 1 antigen is shared by *P. pestis* and *P. pseudotuberculosis,* and this may account for the cross immunity exhibited between these two species. The Oudin technic confirms that Fraction I is the specific antigen for *P. pestis*. Its toxin has cross-reacted with anti-*P. pseudotuberculosis* serum(15), but in a previous study(7) a slight amount of *P. pseudotuberculosis* toxin was not revealed with the anti-*P. pestis* gamma globulin. The *P. pestis* toxin prepared by the paper electrophoresis method in this laboratory has shown 3 bands with anti-*P. pseudotuberculosis* serum and 3 or 4 bands with anti-*P. pestis* gamma globulin. This might be considered a not highly purified toxin.

Virulent strains were not distinguished from avirulent strains by the test.

Summary. *Pasteurella pestis,* virulent or avirulent, contains 7 antigens demonstrable in the Oudin test. Five are common to *P. pseudotuberculosis;* 3 of these are thermolabile and 2 are thermostable. Of the 7 antigens of *P. pestis* 4 are thermolabile and 2 thermostable. The remaining antigen is haptenized Fraction I. Formalin affects all of the antigens except one, rendering them thermolabile; toluene does not affect them.

1. Schütze, H., *Brit. J. Exp. Path.,* 1932, v13, 284.

2. Oudin, J., *Methods in Medical Research,* 1952, v5, 335.

3. Ranson, J. P., Quan, S. F., Hoggan, M., and Omi, G., PROC. SOC. EXP. BIOL. AND MED., 1955, v88, 173.

4. Burrows, T. W., *Mechanisms of Microbial Pathogenicity,* Cambridge University Press, London, 1955, 152-175.

5. Bhagavan, N. V., Nimbkar, Y. S., and Rao, S. S., *Current Sc.,* 1955, v24, 85.

6. Ouchterlony, O., *Acta path. et microbiol. scandinav.,* 1948, v25, 186.

7. Chen, T. H., and Meyer, K. F., *J. Immunol.,* 1955, v74, 501.

8. Girard, G., and Robic, J., *Bull. Office internat.*

d'hyg. pub., 1936, v28, 1078.
9.———, *Bull. Soc. path. exot.*, 1942, v35, 42.
10. Schütze, H., *Brit. J. Exp. Path.*, 1939, v20, 235.
11. Otten, L., *Indian J. M. Res.*, 1936, v24, 73.
12. Chen, T. H., and Meyer, K. F., *J. Infect. Dis.*, 1955, v96, 145.
13. Englesberg, E., and Levy, J. B., *J. Bact.*, 1954, v67, 438.
14. Chen, T. H., Quan, S. F., and Meyer, K. F., *J. Immunol.*, 1952, v68, 147.
15. Chen, T. H., and Meyer, K. F., unpublished data.

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Occurrence of Agglutinogens in Normoblasts.* (22261)

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It is well known that uptake of iron and hemoglobin synthesis occur in the normoblast. There is little information, however, as to the stage of development of the erythrocyte at which agglutinogens first appear. Bjorkman's(1) demonstration that normoblasts may be agglutinated by influenzal virus and a serum containing cold agglutinin suggests that receptors for agglutination make their appearance during this developmental period of the erythrocyte. In his experiments, the normoblasts were isolated from the peripheral blood of a patient with Di Guglielmo's erythro-leukemia; however, the cells were morphologically indistinguishable from myeloblasts. Inasmuch as neoplastic cells could have atypical immunologic characteristics, it seemed desirable to confirm these studies using normoblasts from a patient in which the cells were not of malignant origin.

An opportunity to study agglutinability of normoblasts arose in a patient with autoimmune hemolytic disease secondary to warm agglutinins. About a week following splenectomy, this patient had a hemolytic crisis at which time he released large numbers of nucleated red cells into the peripheral blood. During the period when the normoblast count exceeded 600 per 100 leukocytes, it was a simple matter to isolate them from the peripheral blood for agglutination experiments. A more detailed description of this case will be published elsewhere.

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Methods. To isolate the nucleated red cells, 1.0 ml of a 6% solution of dextran in 0.85% saline was added to 20 cc of the patient's freshly drawn blood in a large siliconized test tube. A 10% solution of sequestrene (disodium ethylenediamine tetracetate) was used as anticoagulant. The mixture was incubated at body temperature for 30 minutes. The supernatant plasma, rich in formed elements, was centrifuged at varying speeds, beginning at 600 rpm for 2 minutes. The residue was resuspended in saline and the supernatant plasma recentrifuged at progressively faster speeds. The resulting cell suspensions were checked for cellular content by means of the phase microscope. It was possible in this way to select test tubes which contained a reasonably pure suspension of normoblasts which were then washed 3 times in saline and prepared as a 4% suspension in 0.85% saline. Suspensions prepared in this way contained a minimal number of contaminating erythrocytes and lymphocytes, but no granulocytes or platelets were present. The normoblast suspension was brought in contact with equal portions of serial saline dilutions of serum from the same patient which contained a potent panhemagglutinin, active at 3°C and 37°C. The normoblast suspension was similarly added to serially diluted serum obtained from another patient with a high titer of cold hemagglutinins. The mixtures were incubated for 12 hours at 3°C and read macroscopically and under the phase microscope. The agglutinating effect of nor-

TABLE I. Agglutination of Normoblasts by Various Sera Obtained from Patient I.B. (Auto-Immune Hemolytic Anemia).

Anti-A serum	P*
Anti-B "	N
Anti-M "	P
Anti-N "	N
Antiglobulin serum	P
I.B. serum (patient)	P
E.S. " (cold agglutinin)	P
E.B. " (" ")	P
Normal A serum	N
B "	P
O "	P

* P = Positive; N = Negative.

mal human sera of various blood groups, in addition to typing sera of the AB and MN and Coombs' antiglobulin serum, were determined following incubation at 37°C for one hour. Siliconized glassware was used in all these experiments.

Results. The reaction of the normoblasts when brought into contact with various sera are listed in Tables I and II. It will be seen that the normoblasts in this patient were agglutinated by anti-A and anti-M serum, which corresponded to his blood type. The normoblasts were strongly agglutinated by antiglobulin serum as were his erythrocytes. Table II lists the titer of serum which resulted in agglutination of the normoblasts. The patient's serum agglutinated his normoblasts in a dilution of 1 to 16 and group O erythrocytes in a dilution of 1 to 8. The serum containing cold agglutinins agglutinated the normoblasts in a dilution of 1 to 256 and normal erythrocytes in a dilution of 1 to 1024.

TABLE II. Titration of Normoblast Agglutinins in Serum of I.B. (Patient) and E.S. (Cold Agglutinin).

	I.B.		E.S.	
Serum dilution	Normoblasts, 3°C	"O" RBC, 3°C	Normoblasts, 3°C	"O" RBC, 3°C
1:1	3+	2+	4+	3+
2	3+	2+	3+	2+
4	3+	1+	3+	2+
8	2+	1+	2+	2+
16	1+	0	2+	2+
32	±	0	1+	2+
64	0	0	1+	2+
128	0	0	1+	2+
256	0	0	1+	2+
512	0	0	±	1+
1024	0	0	0	1+
C	0	0	0	0

Comment. It has been demonstrated in this case that normoblasts derived from a patient with auto-immune hemolytic anemia have antibody receptors which are similar to those of mature erythrocytes. Apparently these receptors make their appearance relatively early in the development of the red cell.

Agglutinability of normoblasts by auto-agglutinins and by antiglobulin serum constitutes direct evidence of the vulnerability of the bone marrow in immune varieties of acquired hemolytic anemia. The severity of anemia in auto-immune hemolytic disease may thus be attributed not only to random destruction of circulating erythrocytes but also may follow a direct attack on proliferating normoblasts. This would limit the ability of the bone marrow to compensate for hemolysis. That impaired erythropoiesis was a factor in hemolytic disease of the new born associated with anti-D antibodies was indicated by the studies of Giblett *et al.*(2). In the case she described, the presence of circulating antibody was correlated with limited erythrocyte production which was resumed following disappearance of anti-D antibodies. In their studies of auto-immune thrombocytopenia, Pisciotta, Stefanini and Dameshek(3) presented morphologic evidence to show that megakaryocytes in a normal recipient could be damaged by transfusion of plasma which contained a strong anti-platelet agglutinin. The damaging effect of the plasma on the megakaryocytes was only temporary and was correlated with a temporarily induced thrombocytopenia.

Summary and conclusions. 1. Normoblasts from a patient with auto-immune hemolytic anemia are agglutinable by type-specific sera, antiglobulin serum and sera containing cold and warm auto-hemagglutinins. 2. Agglutinogens make their appearance at the normoblast stage of development. 3. It is suggested that in acquired immune forms of hemolytic anemia, the bone marrow is also under direct attack by antibodies which may

aggravate the severity of anemia.

1. Bjorkman, S. E., *Acta Haematologica.*, 1954, v11, 189.

2. Giblett, E. R., Varela, J. E., and Finch, C. A., *Pediatrics,* 1956, v17, 37.

3. Pisciotta, A. V., Stefanini, M., and Dameshek, W., *Blood,* 1953, v8, 703.

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Relative Growth-Promoting Activity in Tissue Culture of Co-Factors and the Parent Vitamins. (22262)

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It has been shown(1) that 2 mammalian cells in tissue culture, a human carcinoma cell (strain HeLa) and a mouse fibroblast (strain L), require nicotinamide, pyridoxal, thiamine, riboflavin, pantothenic acid, choline, and folic acid for survival and growth. The degree to which various precursors and conjugates could substitute for the corresponding vitamin in permitting the growth of the mouse fibroblast is here described.

Methods. The technics used in maintaining stock cultures, preparing replicate flasks for feeding with the experimental media, and evaluating the growth response in terms of the number of cells have been described(1-4). As in the previous vitamin experiments(1), specific deficiencies were produced by allowing the cells to grow for varying periods of time (4-14 days) in 1 liter Blake bottles, in the appropriate vitamin-free medium. During this preliminary period of vitamin depletion, the number of cells usually increased 2- to 6-fold in individual experiments; but in most of the experiments, multiplication had then stopped, and there were evidences of cell damage resulting from the vitamin deficiency (cf.(1)). The vitamin-starved cells were resuspended in the appropriate vitamin-free medium, counted, and aliquots of the suspension inoculated into a number of small replicate flasks. Twenty-four hours later, after the cells had adhered to the glass, they were fed with the experimental mixtures containing graded amounts of the vitamin or vitamin substitutes. The flasks were re-fed at 2-day intervals, and the cell count determined after 6-15 days' incubation. Since the cells at the time of the first feeding usually showed cytopathogenic evidence of vitamin depletion, it is apparent that at least 2 factors entered into the results obtained in the present experiments: the capacity of the individual compounds to revive the vitamin-depleted cells, as well as their capacity to support subsequent growth and multiplication. Accordingly, in a second type of experiment, described in the text in relation to folic acid, instead of using vitamin-depleted cells, normal cells were serially propagated in varying concentrations of the vitamin congeners. Diphosphopyridine (DPN) and triphosphopyridine (TPN) nucleotide, flavin adenine mononucleotide (FMN) and dinucleotide (FAD), and cocarboxylase were obtained from the Sigma Laboratories. The first 4 compounds were stated to be 95-98, 60-90, 100, and 65% pure, respectively; but in making stock solutions, all were handled as if they were pure material. Coenzyme A of 75% purity was obtained from the Pabst Laboratory. "Thiamin phosphate" was obtained by autoclaving a solution of cocarboxylase in 0.1 N HCl for 10 min. in a sealed tube. Crystalline pyridoxal phosphate (Palpo) was generously supplied by Dr. E. A. Peterson of the National Cancer Institute, National Institutes of Health.

Results. The results in a number of ex-

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TABLE I. Growth Response of a Mouse Fibroblast (Strain L) to Vitamin Precursors and Conjugates.

	Period of initial starvation in appropriately vit.-deficient medium, days	Degree of cellular multiplication during period of depletion*	Inoculum of vit.-depleted cells, × 10^4	Period of secondary incubation, days	Degree of multiplication* in media containing indicated conc. (M) of specific vitamin, precursor or conjugate								Approximate max effective conc., M
					10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}	10^{-11}	0	
Nicotinic acid	10	2.7	25	6	5.2	5.6	3.0	1.0				.7	10^{-6}
Nicotinamide	10	2.7	25	6	2.6	6.2	4.2	.8				.7	"
DPN	10	2.7	25	6	6.0	6.0	2.2	1.1				.7	"
TPN	10	2.7	25	6	3.1	4.3	2.0	.9				.7	"
Pyridoxine†	8	3.1	12	9		8.8	10	10.8	5.7	.3		.97	10^{-8}
Pyridoxal†	8	3.1	12	9		12	10.4	10.2	8.25			.97	"
Pyridoxamine†	8	3.1	12	9		6.7	10.6	5.0	1.0			.97	10^{-7}
Pyridoxal phosphate†	8	3.1	12	9		9.1	6.4	2.4	1.3			.97	10^{-6}
Thiamin	12	4.1	18	8	7.2	9.3	8.6	8.5	5.8	2.0	.6	.55	10^{-8}
Thiamin phosphate	12	4.1	18	8		2.9	3.7	3.7	3.0	1.7	.2		"
Cocarboxylase	12	4.1	18	8	6.0	7.6	7.5	8.6	3.4	1.2	.6		"
Riboflavin	7	.8	14	7				7.6	9.0	2.8		1.1	10^{-9}
FMN	7	.8	14	7		8.0	13.4	7.6	7.9	3.6	1.3	1.1	"
FAD	7	.8	14	7			15.7	9.5	4.0	2.2	1.5	1.1	10^{-8}
Pantothenic acid	7	1.4	18	15	8.3	9.5	10.9	11	.33			0	"
CoA	7	1.4	18	15	7.4	7.9	7.1	.14				0	10^{-7}
Folic acid	14	3.9	16	6		10.6	9.6	4.6	2.0	2.1		.6	10^{-7} to 10^{-8}
Citrovorum factor (natural)†	14	3.9	16	6		11	10.8	10.8	9.2	2.2		.6	10^{-9}†
PABA	14	3.9	16	6		.9		.8		.8		.6	

* Referred to inoculum as 1. † Gravimetric concentration (g/ml) rather than molar.

periments with the mouse fibroblast are given in Table I. Each experiment there shown is illustrative of several others, with qualitatively similar results.

1. *Nicotinamide,* nicotinic acid, DPN and TPN all permitted the growth of mouse fibroblasts previously depleted of the vitamin by 10 days' starvation. Although the 4 compounds were equally active in terms of their effective concentrations, more growth was usually obtained with nicotinamide and DPN than with nicotinic acid or TPN.

2. *Pyridoxal* and pyridoxine were essentially equivalent in their growth-promoting activity. Although pyridoxamine and pyridoxal phosphate could substitute for pyridoxal, with the preparations here used somewhat higher concentrations were necessary.

3. *Thiamine* and cocarboxylase proved equivalent, both with respect to the concentrations required for maximal growth, and the amount of growth obtained at that optimal concentration. Smaller amounts of growth were, however, observed with "thiamine phosphate."

4. *Riboflavin* and FMN proved equivalent in growth-promoting activity. With FAD, however, significantly higher concentrations were required for a comparable effect.

5. *Pantothenic acid* proved approximately 10 times more active than the 2 samples of coenzyme A used in these experiments (Fig. 1). It is of interest that a similar quantitative difference in the activity of pantothenic acid and coenzyme A was noted by Dewey and Kidder(5) for *Tetrahymena pyriformis* and *Colpidium campylum.*

6. *Folic acid* was regularly less active than natural citrovorum factor in terms of the amounts required for the revival and growth of the vitamin-depleted cells. p-Aminobenzoic acid (PABA) proved wholly inactive (Fig. 1).

To determine whether PABA would permit the survival and growth of normal cells, not previously depleted of folic acid or its derivatives, normal cells, not previously damaged by folic acid depletion, were serially propagated in varying concentrations of folic acid and of PABA. Even under these circumstances, however, PABA failed to permit the continuing growth of the cells in the absence of folic acid.

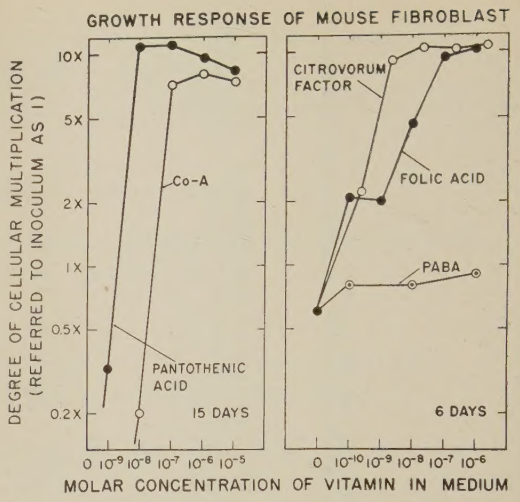

FIG. 1. Varying growth response of mouse fibroblast. (a) Pantothenic acid and coenzyme A; (b) Citrovorum factor, folic acid and PABA.

Summary. A number of the co-factors here tested (FMN, DPN, cocarboxylase) had essentially the same activity as the corresponding vitamin (riboflavin, nicotinamide and thiamine, respectively) in promoting the growth of the mouse fibroblast, in terms of either the effective concentration or the amount of growth obtained. Several vitamin conjugates (FAD, TPN, coenzyme A and pyridoxal phosphate) proved significantly less active than the parent vitamin (riboflavin, nicotinamide, pantothenate, and pyridoxal, respectively). The present experiments provide no information as to the degree to which these differences may reflect only variations in cell permeability, rather than partial blocks in the utilization of these specific co-factors, or in their conversion to a more active form. Natural citrovorum factor, however, was somewhat more active than folic acid. Of the vitamin congeners or precursors here tested, pyridoxine and pyridoxal were essentially equivalent in activity; nicotinic acid was significantly less active than either nicotinamide or DPN in terms of the amount of growth obtained, although the effective concentrations were of the same order of magnitude; while PABA was wholly inactive as a substi-

tute for folic acid. As with the co-factors, the present experiments do not exclude differential cell permeability as the basis of these differences.

1. Eagle, H., *J. Exp. Med.*, 1955, v102, 595.
2. ———, *J. Biol. Chem.*, 1955, v214, 839.
3. ———, *J. Exp. Med.*, 1955, v102, 37.
4. Eagle, H., Oyama, V. I., Levy, M., Horton, C. L., and Fleischman, R., *J. Biol. Chem.*, in press.
5. Dewey, V. C., and Kidder, G. W., PROC. SOC. EXP. BIOL. AND MED., 1954, v87, 198.

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Viral Susceptibility of a Human Carcinoma Cell (Strain KB). (22263)

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The cultivation of a human epidermoid carcinoma of the floor of the mouth (strain KB) by direct implantation in a fluid medium has been described(1). As will be here shown, this cell line has proved susceptible to a number of human and animal viruses.

Methods. The basal medium used in these experiments was the same as that used in the original isolation, and consisted of the amino acids, vitamins and salts essential for the growth of the HeLa cell, at the concentrations optimal for that cell(2-5). In the initial cultivation this was supplemented with 10% whole human serum. In the present experiments, the same basal medium was supplemented with 4% whole horse serum, in order to avoid the complication introduced by the presence in pooled human serum of antibodies to some of the viruses. Four-day cultures of the cell line in T-15 flasks(2), then containing approximately 4.5 x 10^6 cells, were washed twice with the horse serum medium, and refed with 2.5 ml of fresh medium. One-tenth ml of the viral suspensions of Table I, diluted as there indicated, were then added. The medium was then replaced at 2-day intervals. This first passage test was terminated 7 or 8 days after the original inoculation. The times required for the earliest cytopathogenic effect, and for the destruction of more than 75% of the cells, are indicated in Table I. Fluids harvested at the time of maximal cytopathogenic effect (or after 7 to 8 days in the case of those viruses with no apparent effect) were then similarly inoculated into somewhat larger (T-30) culture flasks containing approximately 4.2 x 10^6 cells at time of inoculation. The virus inoculum in this second passage was 0.5 ml in a total of 5 ml. As in the first passage, the fluids were changed every other day, beginning the day after inoculation. The time required for the earliest visible cytopathogenic effect, and for the almost total destruction of the cells is shown for each virus in the last 2 columns of Table I. Material harvested from this second passage at the time of maximal cytopathogenic effect (or after 6 days in the case of those viruses with no apparent effect) was assayed for viral content by either complement-fixation, pathogenicity in tissue culture, or animal inoculation. The results of those tests are summarized in Table II.

Results. Cytopathogenic effects were produced in the KB cell in both first and second passages by type 1 poliomyelitis (Mahoney), herpes simplex, vaccinia, types 1, 3, and 4 adenoidal-pharyngeal-conjunctival (APC) viruses, lymphocytic choriomeningitis (LCM), and encephalomyocarditis (EMC) viruses (*cf.* Tables I and II). In all these, the titers of the second passage fluids indicated that the viruses had multiplied. The titer of complement-fixing antigen produced by the type 1, 3 and 4 APC viruses was significantly higher than had previously been obtained in

* Public Health Service, U. S. Department of Health, Education and Welfare.

TABLE I. The Susceptibility of the KB Cell to a Number of Human and Animal Viruses.

		Primary inoculation*			Second passage†		
Virus	Source of virus	Final dilution of viral suspension in culture medium	Days for beginning of cytopathogenic effect	Days for more than 75% of cells to degenerate	Harvested from first passage on day No.	Days for beginning of cytopathogenic effect	Days for more than 75% of cells to degenerate
Poliomyelitis (Mahoney)	Monkey kidney culture	1: 100	1	2	2	1	1
Herpes simplex	Adult mouse brain	"	2	4	4	1	2
Vaccinia	HeLa culture	1: 250	1	2, 3	2, 4, 5 (pool)	1	2
APC, type 1	*Idem*	"	4	6	6	1	2
3	"	"	4–6	>8‡	8	1	1
4	"	"	3–4	5–6	6	1	1
Mumps	Suckling mouse brain	1:1000	4	7	7	2	3
"	Allantoic fluid	1: 100	2	3	7	2	3
LCM	Adult mouse brain	1:1000	3	7	7	3	5
EMC	*Idem*	"	1	3	3	6	6
Rabies	Adult mouse brain	1:1000	4	5	7	>6‡	—
GD VII	*Idem*	"	6	>7‡	7	>6	—
Yellow fever	"	"	5±	>7	7	>6	—
APC (SV_1) (6)	Monkey kidney culture	1: 25	1	1–2	4	>6	—
(DC) (7)	HeLa culture	1: 10	6(?)	>8	8	>6	—
(Type 8) (8)	*Idem*	"	6(?)	>8	8	>6	—
Coxsackie A9	Monkey kidney culture	1: 250	>8‡	>8	8	>6	—‡
Influenza (type B)	Allantoic fluid	1: 100	>8	>8	7	>6	—
Idem	Monkey kidney culture	"	6(?)	>7	7	>6	—

* In T-15 flasks containing 450 × 10^4 cells, and 2.5 ml fluid. Culture fluid changed every 2 days (days #2, 4, 6, 8 in some, and 1, 3, 5, 7 in others) unless cells had degenerated in interim.

† In T-30 flasks containing 420 × 10^4 cells, and 5 ml fluid. Culture fluid changed on day #1, 3, 5 (6) unless cells had degenerated in interim.

‡ No cytopathogenic effect at the time indicated.

HeLa cell cultures. In subsequent experiments, APC viruses of types 1-6 were carried through 5 serial passages in KB cells, and herpes simplex and vaccinia viruses through 3 passages, with rapid production of cytopathogenic effects in all passages. Isolations of all 3 types of poliomyelitis and of several types of APC viruses from field specimens have also been made in KB cells.

Equivocal results were obtained with 4 additional viruses. a) Rabies virus produced cytopathogenic changes in the primary inoculation, but not in the second passage, and no virus could be recovered from the latter. b) The SV_1 strain of APC(6) similarly produced cytopathogenic changes on primary inoculation, and not in second passage. However, the second passage material contained virus, demonstrable by cytopathogenic effect in monkey kidney; and on continued passages through the KB cell, virus was detectable after the fourth passage, indicative of continuing viral multiplication in the absence of significant cytopathogenic effect. c) Both strains of mumps virus produced cytopathogenic effects in both the first and second passage, but did not cause the death of the culture. Cellular degeneration and multiplication proceeded simultaneously throughout the period of test. There is no information as to whether the elaboration of virus in such cultures (*cf.* also SV_1 and yellow fever in this group) reflected the production of virus by only a portion of the cells, which degenerated

TABLE II. Evidence of Viral Multiplication in KB Cell.

Virus	Viral assay of original inoculum*		Viral activity* of 2nd passage harvest				
	Tissue culture	Animal or egg inoc.	Day on which harvested	Estimated dilution of original inoculum in harvest†	Complement fixing titer of antigen	Tissue culture titer	Animal or egg inoculation†
Poliomyelitis (Mahoney)	7.5		1	$1:10^4$		8.5	
Herpes simplex		4.0	2	"			4.8
Vaccinia		‖	3	"		>2.5	
APC, type 1	7.0		3	$1:2 \times 10^5$	1:256	9.5	
" " 3	8.0		1	$1:2 \times 10^6$	1: 32	8.0	
" " 4			2	$1:5 \times 10^5$	1:128	8.0	
Mumps (mouse)		4.5	5	$1:10^8$			>4.0
LCM		5.0	5	$1:10^9$			5.5
EMC		8.6	6	$1:10^6$			>8.0
Yellow fever		6.0	6	$1:10^9$			2.5
APC (SV_1) (6)	6.5		6	$1:2 \times 10^6$		2.5	
Rabies		7.5	6	$1:10^9$			<1.5§
GD VII		7.0	6	"			<1.5§
APC (DC) (7)		‖	6	$1:10^8$	<1:4	<1.0	
" (Type 8) (8)		‖	5	"	"	1.5	
Coxsackie A9	7.0±		5	$1:5 \times 10^8$		ca. 1.5	
Influenza (type B)		7.0	6	$1:10^8$			<1.5§
Idem		3.0	6	"			<1.5§
Mumps (egg)		7.5	5	$1:10^7$			<1.5§

* Logarithm of infectious doses per ml.

† Based on amounts of inoculum introduced into flask, and dilution effected by repeated fluid changes, on the assumption that up to 10% of fluid may have been left each time medium was changed as indicated in footnotes of Table I.

‡ Intracerebrally in adult mice, except for (a) mumps virus, inoculated intracerebrally in suckling mice, and (b) influenza virus, inoculated into allantoic sac of egg embryos.

§ Negative in highest concentration tested.

‖ Original inoculum not titered.

in consequence, or whether even the cells which showed no significant cytopathogenic changes were participating in the elaboration of virus. When the second passage material of the mumps cultures was tested intracerebrally in suckling mice, only the culture of the mouse-derived strain proved infectious; and neither strain was now infectious in eggs. In a following experiment, mumps virus was passaged 5 times. In each passage there was a 5% inoculum, the fluid was changed every 2 days, and the harvest for sub-inoculation was collected on the 6th day. The cytopathogenic effect varied markedly and irregularly for individual passages, from no demonstrable change in the 2nd and 3rd passage, to approximately 50-75% degeneration in the 1st and last passages. The 5th passage harvests were estimated to represent a 10^{-15} dilution of the original inoculum. As in the previous experiment, when the 5th passage material was inoculated intracerebrally in suckling mice, only the culture of the mouse strain was infectious; and neither strain could now produce either infection or demonstrable virus in eggs. There was thus definite multiplication of the mouse-adapted mumps strain; but the only indication that the egg-adapted strain had propagated was the partial and irregular cytopathogenicity. d) Although yellow fever virus produced no significant cytopathogenic effect in either passage, the second passage material proved infectious on intracerebral inoculation in mice. In a second experiment, after 5 passages through the KB cell, estimated to represent a 10^{-15} dilution of the original inoculum, the undiluted culture material produced infection on intracerebral inoculation in mice. As with the mumps strains, the cytopathogenic effects in these serial passages were irregular and incomplete.

The other viruses studied, including mouse

encephalomyelitis (Theiler GD VII), 2 other APC strains (type 8 and strain DC), a Coxsackie virus, and influenza type B, produced no cytopathogenic effects in either passage, and except for a small amount of residual virus in the second passage of the APC type 8 and Coxsackie A9, there was no evidence of viral multiplication. In subsequent attempts with higher titer inocula, type 8 APC has been established in serial passage. It should be pointed out that the DC strain grows poorly in all cell types tested in tissue culture, including HeLa cells(7).

Summary. 1) A human epithelioid carcinoma cell in tissue culture (strain KB) has been found susceptible to a number of viruses, including poliomyelitis, herpes simplex, vaccinia, a number of APC strains, LCM and EMC. Marked cytopathogenic effects were produced in 1-7 days, and viral multiplication was shown by complement fixation, tissue culture, or animal titration of the second passage material. 2) Only partial and irregular cytopathogenic effects were obtained with mumps, yellow fever, and the SV_1 strain of APC virus, but with apparently continuing elaboration of virus on repeated passage. Both an egg-adapted and a mouse strain of mumps virus were found to have lost their infectivity for eggs after several culture passages. 3) Coxsackie virus (strain A9), influenza type B, one APC type, and GD VII had no cytopathogenic effect on this cell, and there was no evidence of viral multiplication. Rabies virus was cytopathogenic in the first passage only; and the harvest of the second passage was not infectious on intracerebral inoculation in mice.

1. Eagle, H., PROC. SOC. EXP. BIOL. AND MED., 1955, v89, 362.

2. ———, *J. Biol. Chem.*, 1955, v214, 839.

3. ———, *J. Exp. Med.*, 1955, v102, 37.

4. ———, *ibid.*, 1955, v102, 595.

5. Eagle, H., Oyama, V. I., Levy, M., Horton, C. L., and Fleischman, R., *J. Biol. Chem.*, 1956, v218, 607.

6. Hull, R. N., Minner, J. R., and Smith, J. W., to be published.

7. Rowe, W. P., Hartley, J. W., and Huebner, R. J., PROC. SOC. EXP. BIOL. AND MED., 1956, v91, 260.

8. Jawetz, E., Kimura, S., Nicholas, A. N., Thygeson, P., and Hanna, L., *Science*, 1955, v122, 1190.

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Comparative Absorption of Vitamin B_{12} Analogues by Normal Humans. II. Chloro-, Sulfato-, Nitro- and Thiocyanato- *vs* Cyanocobalamin. (22264)

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The first(1) paper of this series reported results of comparative oral absorption studies of chlorocobalamin and cyanocobalamin employing the urinary excretion method of Schilling(2). These tests were interpreted as indicating the superiority of cyanocobalamin over chlorocobalamin for alimentary absorption by humans. Results of oral tolerance tests with humans, and of fecal excretion measurements with rats, confirming the low chlorocobalamin absorption, are reported herein. In addition, similarly inferior absorptions of sulfato-, nitro- and thiocyanatocobalamins are reported, based upon urinary and fecal excretion studies in humans, and upon fecal excretion by rats.

Methods. Materials. The preparation and properties of the radioactive and normal chlorocobalamins and cyanocobalamins employed in these experiments have been described(1). Sulfatocobalamins were obtained photochemically as for chlorocobalamin except that irradiation was performed in 0.01 M H_2SO_4 solution. Samples of the nitroco-

TABLE I. Urinary Excretion of Radioactivity by Humans.

Cobalamin			Avg % of dose
Oral		Inj., 1 mg	± stand. dev.
Cyano	2 μg	Cyano; chloro	≃12*
Sulfato	"	Sulfato	3.5 ± 1.5
		Chloro	2.6 ± .8
		Cyano	3.7 ± 1.8
Nitro	"	Nitro	2.0 ± .8
		Chloro	3.1 ± .5
		Cyano	1.9 ± .2
Thiocyanato	"	"	2.0 ± .9
Nitro	.5	"	5.8 ± 3.6
Cyano	"	"	17.6 ± 9.0

* Avg of accumulated standard test data.

balamins and thiocyanatocobalamins were prepared from sulfatocobalamins by treatment with acid nitrite and KSCN respectively. Paper chromatograms,* distribution coefficients and absorption spectra attested to the identity of the radioactive and normal forms. Specific activities of the radioactive cobalamins were: cyano-≃200 μc/mg; sulfato-≃130 μc/mg; nitro-≃130 μc/mg and thiocyanato-≃171 μc/mg. The urinary excretion(2,3) and the Heinle-Welch(4) fecal excretion (5-6 days) methods employed to compare the several labeled cobalamins have been adequately described elsewhere. In all cases the subjects (groups of 4-6) were young, healthy adult males under continual supervision to insure complete collection of excreta. Urine and feces radioactivity was determined by scintillation counting. Two series of oral tolerance tests were performed with chloro- and cyanocobalamin as described by Chow(5). Subjects were fed 3 or 5 mg of test substance in aqueous solution after first removing 15 ml of fasting blood for microbiological assay of serum for vitamin B_{12}. Two hours after ingestion, another 15 ml sample of blood (still fasting) was removed for vit. B_{12} assay. Aged subjects, *i.e.* over 65 years, were employed in order to reveal more readily resultant changes in serum vit. B_{12}. Only differences in excess of 40 μμg/ml are considered experimentally significant(5).

* The authors are grateful to R. D. Babson of the Research Laboratories of Merck & Co., for these chromatographic tests.

Normal adult male rats of the Johns Hopkins colony, weighing an average of 300 g per animal, were employed (in groups of 6) in fecal excretion experiments with cyano-, chloro- and nitrocobalamin-Co^{60}. A 50 mμg dose of labeled analogue was fed to each rat by stomach tube and feces collected for 5 days. All collections were measured for cobalt 60 content by scintillation counting of feces homogenates. Actually, excretion of radioactivity was negligible after the first 24–48 hours.

Results. Human urinary excretion results are compiled in Table I. Table II contains the results of 2 fecal excretion studies with 0.5 μg of cyano- and nitrocobalamin-Co^{60} administered to human subjects. Although daily measurements were performed, only cumulative excretions for the entire collection periods are of interest. Group average cumulative excretions measured, and average absorption values computed therefrom by difference, are reported.

Results of the 2 oral tolerance tests are compiled in Table III. Initial concentrations and maximum difference, observed after 2 hours, are reported in terms of μμg/ml of serum.

TABLE II. Fecal Excretion of Radioactivity by Humans.

Study	Cobalamin (.5 μg oral)	Avg % excr. ± stand. dev.	Avg % absorbed
1st* (5 days)	Cyano	18.0 ± 7.1	82.0
	Nitro	41.8 ±12.8	58.2
2nd (6 days)	Cyano	13.7 ± 4.4	86.3
	Nitro	46.5 ± 6.9	53.5

* 1 mg vit. B_{12} injected 2 hr after oral dose of tracer.

TABLE III. Serum Levels (μμg/ml) in Humans.

	Cyano		Chloro	
Study	Initial	Difference (2 hr)	Initial	Difference (2 hr)
1st	280	682	222	0
3 mg cyano;	146	854	216	0
3 mg chloro	251	70	157	0
	169	286	642	0
	227	94	570	305
2nd	99	204	169	76
3 mg cyano;	122	158	140	41
5 " chloro	157	141	251	46
	227	94	490	0
	198	64	146	0

TABLE IV. Fecal Excretion of Radioactivity by Rats.

Cobalamin (.050 μg oral)	Avg % excr. ± stand. dev.	Avg % absorbed
Cyano	65.4 ± 7.2	34.6
Chloro	90.6 ± 9.5	9.4
Nitro	85.2 ± 15.2	14.8

Table IV is a summary of the findings from the fecal excretion study with cyano-, chloro- and nitro-cobalamin-Co^{60} administered to rats. Here too, group average cumulative excretions (after 5 days) and computed absorptions, are reported.

Discussion. Application of the urinary excretion method at 2 μg to sulfato-, thiocyanato- and nitrocobalamin reveals lower responses than are obtained with cyanocobalamin, as can be seen from the summary Table I. For nitrocobalamin, this is true also at the 0.5 μg oral level. The low sulfato-excretions (≃3% of the 2 μg dose) are practically identical with those reported previously for the chloro-analog, and are about one-fourth the average 12% figure typical of vit. B_{12} itself. Similar behavior characterizes nitrocobalamin and thiocyanato cobalamin *i.e.* low responses of 1.9-3.1% of the 2 μg dose independent of the choice of injected analogue. Even at 0.5 μg oral intake, the urinary nitrocobalamin output of 5.8% is much less than the 17.6% elimination of cyanocobalamin. Furthermore as in the case of labeled chloro- (1), the nature of the injected cobalamin is immaterial. Thus sulfato-, chloro-, nitro- and cyano-cobalamin are indistinguishable in their capacity to induce cobalamin excretion upon injection, which again points to the equivalence, perhaps through interconvertibility of cobalamins or conversion to another common structural analogue, of the several cobalamins once introduced into the blood stream.

The similarity in behavior of the chloro- and sulfato-forms is understandable in terms of their identical electrolyte and hydrophilic properties(6,7) and spectra. Less evident is the reason for the slight responses obtained with nitrocobalamin and thiocyanato-cobalamin. These compounds are characterized as electrically neutral cobalamins(7,8) since the substituent anions are relatively tightly bound(9) and because of their relative organophilic tendencies(9,10). In this respect they resemble cyanocobalamin rather than aquocobalamin salts, and might have been expected to exhibit a pronounced absorption closely approximating that of vit. B_{12} itself. Despite evidence for slight dissociability in certain cases, addition of cyanide ion converts all known cobalamins to the cyano form. The current results then point to the unique importance of the latter group in governing the metabolic activity of the cobalamins, rather than to the dissociability of the substituent anion. It is conceivable that even the low non-cyano responses are themselves due to partial interactions with traces of cyanide ion in gastric juice or upper intestinal contents rather than to an inherent activity of these cobalamins.

That the extent of urinary excretion after injection is an index of absorption has been verified by independent methods of demonstrating absorption.† In the case of chlorocobalamin, oral tolerance tests (Table III; first study) with humans failed to reveal any increase in serum cobalamin concentration in four out of five subjects fed 3 mg doses; and even feeding of 5 mg (Table III; second study) produces a barely significant rise in 3 out of 5 persons. By contrast, ten individuals receiving 3 mg of cyanocobalamin each exhibited serum levels 2 hours after ingestion which were on the average approximately 2-3 times normal. Further evidence for the superior absorption of cyanocobalamin is provided by the fecal excretion measurements with rats. It is obvious from Table IV that the absorption (9.4% of the 50 mμg oral dose) of chlorocobalamin is only about one-fourth the 34.6% absorption typifying cyanocobalamin.

Similar confirmation has been obtained in

† G. B. J. Glass, N. Y. Medical College has observed(11) by hepatic uptake method that the cpm of liver projections after oral administration of 0.5 μg of chlorocobalamin-Co^{60} is only one-half to one-third the radioactivity noted after administration of cyanocobalamin-Co^{60}. This is in accord with conclusions presented in our paper.

studies comparing the fecal excretion of labeled nitrocobalamin with that of cyanocobalamin by rats and humans. In the rat studies (Table IV) nitrocobalamin absorption (14.8%) is less than half the 34.6% value obtained with the cyano- form. In the first of the human studies (Table II), fecal excretions of nitro- and cyano- forms were respectively 41.8% and 18.0% of the 0.5 μg oral dose which presumably correspond to absorptions of 58.2% and 82.0%. Respective absorption figures of 53.5% and 86.3% obtained in the second study are in excellent agreement. When one considers that "zero" absorption values, as are obtained with pernicious anemia patients(12), may range from 0 to 24% (average≃12%), these figures would indicate a 2 to 1 superiority in absorption in favor of cyanocobalamin.

It is significant that all methods support the observations, based upon the urinary excretion method, that cyanocobalamin excels the other cobalamins as an oral absorption form. One may conclude further that, as regards the cobalamin family, the injection-excretion test can serve as an index by which to judge comparative absorption.

Summary. 1. Urinary excretion measurements indicate considerably lower oral absorption of sulfato-, nitro- and thiocyanatocobalamin than is exhibited by cyanocobalamin. In the blood stream, however, the several cobalamins are equivalent in inducing the excretion of ingested vit. B_{12}. 2. That cobalamin urinary excretion responses truly constitute an absorption index was verified in studies comparing chlorocobalamin with cyanocobalamin, by serum level assays in humans and by fecal excretion experiments with rats. 3. Inferior absorption of orally administered nitrocobalamin was confirmed by fecal excretion observations in humans and rats. 4. The superior absorption of cyanocobalamin (vit. B_{12}) over other cobalamins studied is attributed to either the chemical specificity of the substituent cyano group or the tightness of its binding in the cobalt coordination sphere.

1. Rosenblum, C., Woodbury, D. T., Gilbert, J. P., Okuda, K., and Chow, B. F., PROC. SOC. EXP. BIOL. AND MED., 1955, v89, 63.

2. Schilling, R. F., *J. Lab. Clin. Med.,* 1953, v42, 860.

3. Chow, B. F., Gilbert, J. P., Okuda, K., and Rosenblum, C., *Am. J. Clin. Nutrition,* in press.

4. Heinle, R. W., Welch, A. D., Scharf, V., Meacham, G. C., and Prusoff, W. H., *Trans. Assn. Am. Physicians,* 1952, v65, 214.

5. Chow, B. F., *Symposium on Problems of Gerontology,* National Vitamin Foundation, New York, Aug. 1954, p59.

6. Kaczka, E. A., Wolf, D. E., Kuehl, F. A., Jr., and Folkers, K., *J. Am. Chem. Soc.,* 1951, v73, 3569.

7. Smith, E. L., Ball, S., and Ireland D., *Biochem. J.,* 1952, v52, 395.

8. Heinrich, H. C., and Lahann, H., *Zeits. Vitamin-, Hormon- Fermentforschung,* 1954, vVI, [No. 2-3], 126.

9. Smith, E. L., Fantes, K. H., Ball, S., Waller, J. G., Emery, W. B., Anslow, W. K., and Walker, A. D., *Biochem. J.,* 1952, v52, 384.

10. Buhs, R. P., Newstead, E. G., and Trenner, N. R., *Science,* 1951, v113, 625.

11. Glass, G. B. J., personal communication.

12. Callender, S. T., Turnbull, A., and Wakisaka, G., *Brit. Med. J.,* 1954, v1, (No. 4852), 10.

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Decrease of Coenzyme A Content in Fatty Liver.* (22265)

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Some years ago it was found that pantothenic acid deficiency produces fatty liver in dogs(1). This observation appeared of particular interest after the discovery of coenzyme A (CoA)(2) and the identification of pantothenic acid as precursor of CoA(3,4), this coenzyme having a pivotal position in degradation and synthesis of fatty acids and cholesterol(5-7). Since CoA represents the functional form of pantothenic acid in tissues, it appeared reasonable to suppose some relation between CoA-deficiency and formation of fatty liver. However, more recently it has been reported that, not only does fatty liver not occur in pantothenic acid-deficient rats but that these rats are resistant to liver fat deposition ordinarily caused by cholesterol-rich diet(8). The difference of results in the 2 species is probably due to the fact that adrenal changes produced by pantothenic acid deficiency are much more severe in rats than in dogs(*cf.* 9). Adrenal hypofunction has been found to occur in pantothenic acid-deficient rats, which, in some respects, react as though adrenalectomized(10). That adrenal glands play an important role in the formation of fatty liver is shown by the fact that adrenalectomy prevents deposition of fat in the liver due to different steatogenic treatments(11-16). It seemed to us worthwhile to investigate whether CoA is implicated in the pathological accumulation of fat in liver. This paper reports the results of the estimation of CoA in fatty liver produced with carbon tetrachloride.

Materials and methods. Adult female albino rats, weighing between 250-300 g, were used. Animals were kept on a standard diet of the following composition: glucose, 73%; casein, 18%; maize oil, 3%; cod liver oil, 2%; salts, 4%(17). Supplementary crystalline vitamins were added in the following quantities per 100 g of diet: thiamine, 400 μg; riboflavin, 800 μg; pyridoxine, 400 μg; niacin, 2000 μg; calcium pantothenate, 2000 μg and choline chloride, 100 mg. After 3 weeks on this diet, the animals were divided into 2 groups. Rats of the first group were used as normal controls and those of the second group were injected for 8 days with CCl_4 to produce fatty liver (0.2 ml/100 g body wt/day of 20% solution of CCl_4 in olive oil, subcutaneously). During treatment with CCl_4 the animals were fed with the aforementioned standard diet. Both normal and experimental rats were fasted for 12 hr before the experiment. They were killed by decapitation and livers were quickly removed. Portions of liver were taken for histological examination and for estimation of nitrogen and lipid contents. The remainder of hepatic tissue was immediately chilled and homogenized with water. The homogenate was rapidly boiled, centrifuged, filtered(18) and CoA estimated in the boiled extract by a modification(19) of the method of Kaplan and Lipmann(18). The tissue samples for histological study were placed in 10% formalin within a few minutes after death; paraffin sections were stained with hematoxylin-eosin and frozen sections were stained with Sudan III for fat and hematoxylin as a counterstain. Nitrogen was determined with Nessler's reagent after digestion of the samples with sulphuric acid containing copper selenite(20). Total lipids were determined by extraction of dry tissue with ether in Soxhlet apparatus and weighing of fatty residue after removal of solvent. Tissue was dried by heating in oven at 100°C until constant weight. Acetylating apoenzyme was prepared from pigeon livers according to Kaplan and Lipmann(18) but livers were frozen overnight and homogenized directly from the frozen state into cold acetone as suggested by Handschumacher, Mueller and Strong(19). Chromatographically pure 4-aminoazobenzene was used; the commercial product ob-

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TABLE I. Coenzyme A Content in Normal and Fatty Livers.*

	Total lipids, mg/mg liver N.	CoA, μg pantothenic acid bound as Co-A/mg liver N.
Normal rats	1.32 ± .07	2.2 ± .13
CCl_4-intoxicated rats	2.41 ± .11	1.54 ± .11
Difference of the means	1.09	.66
P†	<.01	<.01

* Figures are means of 8 experiments ± stand. error.

† Probability determined by *t* test.

tained from Eastman Kodak Co. was purified as indicated by Handschumacher *et al.*(19). Adenosine triphosphoric acid (barium salt) and a crude preparation of CoA were obtained from the Sigma Chemical Co. All the other chemicals were reagent grade.

Results. Microscopic examination confirmed that CCl_4-intoxicated rats developed typical fatty livers. Chemical analyses of livers revealed amounts of lipid which corresponded well with the microscopic picture. As to be expected, the fat content was markedly increased in livers of CCl_4-intoxicated animals (Table I). The results of CoA estimations in both normal and pathological livers are summarized in Table I. It is apparent that CoA content was significantly lowered in fatty livers as compared with normal controls (30% decrease).

Discussion. Results presented are sufficient evidence for a marked decrease of CoA content in fatty liver. However, because of the method used in estimation of CoA it is possible that the actual decrease of CoA in fatty liver was greater than we observed. It has been shown that pigeon liver extract of Kaplan and Lipmann(18), in the presence of adenosine triphosphate (ATP) is capable of synthesis of CoA from some fragments containing pantetheine, in particular from dephospho-CoA and from phosphopantetheine (21,22). The CoA-assay system used in our experiments included both ATP and the Kaplan-Lipmann extract of pigeon liver as an acetylating apoenzyme. Thus, values for CoA here reported represent the sum of preexisting complete CoA plus any CoA synthesized from smaller molecules during the assay. The possibility exists that the amount of precursors of CoA was increased in fatty liver because of an impaired formation of complete coenzyme. If this were true, the synthesis of CoA during the assay would erroneously raise the figures for fatty livers more than those for normal livers. The uncoupling of oxidative phosphorylation found in mitochondria from fatty livers(23) could account for the impairment of CoA synthesis and for the accumulation of the precursors. ATP, in fact, is involved in biosynthesis of CoA(24). It is also to be noted that mitochondria swollen *in vitro* release CoA into the suspension medium where the coenzyme is broken down to fragments containing pantetheine(25). This observation may be of interest because mitochondria from fatty liver closely resemble, in their morphology and enzymatic activities, osmotically damaged mitochondria(23). Although an impaired synthesis seems the most likely explanation for the decreased content of CoA in fatty liver, the possibility of an increased breakdown of the coenzyme cannot be ruled out. It also remains obscure whether the CoA-deficiency plays a pathogenic role in the formation of fatty liver or it only represents a consequence of cell damage. As far as concerns the possible participation of CoA-deficiency in the pathogenesis of fatty liver, a hypothesis can be put forward if only provisionally. The low level of CoA and ATP, which are necessary for activation of fatty acids, would lead to impaired utilization of lipids in the liver and, consequently, to pathological accumulation of fat. Of course, this pathological condition is more severe when fat mobilization from depots to the liver is accelerated. According to the results of previous work(*cf.* 26), an increased mobilization of fat from the peripheral tissues to the liver actually occurs during fatty liver formation.

Summary. Coenzyme A has been estimated in both normal and CCl_4-fatty livers. The level of coenzyme A is decreased in fatty livers as compared with normal controls. The possible role of this change in the formation of fatty liver is discussed.

We wish to thank Professor E. Ciaranfi for his interest in this work.

1. Schaefer, A. E., McKibbin, J. M., and Elvehjem, C. A., *J. Biol. Chem.,* 1942, v143, 321.

2. Lipmann, F., *ibid.,* 1945, v160, 173.

3. Lipmann, F., Kaplan, N. O., Novelli, G. D., Tuttle, L. C., and Guirard, B. M., *ibid.,* 1947, v167, 869.

4. Novelli, G. D., Kaplan, N. O., and Lipmann, F., *ibid.,* 1949, v177, 97.

5. Lipmann, F., *Bact. Rev.,* 1953, v17, 1.

6. Klein, H. P., and Lipmann, F., *J. Biol. Chem.,* 1953, v203, 101.

7. Lynen, F., *Exposés Ann. Bioch. Méd.,* 1954, Ser. 16, 31.

8. Guehring, R. R., Hurley, L. S., and Morgan, A. F., *J. Biol. Chem.,* 1952, v197, 485.

9. Novelli, G. D., *Physiol. Rev.,* 1953, v33, 525.

10. Hurley, L. S., and Morgan, A. F., *J. Biol. Chem.,* 1952, v195, 583.

11. Verzár, F., and Lastz, L., *Biochem. Z.,* 1936, v288, 356.

12. v. Issekutz, B., Jr., and Verzár, F., *Arch. Ges. Physiol.,* 1938, v240, 624.

13. MacKay, E. M., and Barnes, R. H., *Am. J. Physiol.,* 1937, v118, 525.

14. Long, C. N. H., and Lukens, F. D. W., *ibid.,* 1936, v116, 96.

15. ———, *J. Exp. Med.,* 1936, v63, 465.

16. MacKay, E. M., and Carne, H. O., PROC. SOC. EXP. BIOL. AND MED., 1938, v38, 131.

17. Hegsted, D. M., Mills, R. C., Elvehjem, C. A., and Hart, E. B., *J. Biol. Chem.,* 1941, v138, 459.

18. Kaplan, N. O., and Lipmann, F., *ibid.,* 1948, v174, 37.

19. Handschumacher, R. E., Mueller, G. C., and Strong, F. M., *ibid.,* 1951, v189, 335.

20. Umbreit, W. W., Burris, R. H., and Stauffer, J. F., *Manometric Techniques and Tissue Metabolism,* Burgess Publ. Co., Minneapolis, 1949.

21. Novelli, G. D., Kaplan, N. O., and Lipmann, F., *Fed. Proc.,* 1950, v9, 209.

22. Novelli, G. D., Schmetz, F. J., and Kaplan, N. O., *J. Biol. Chem.,* 1954, v206, 533.

23. Dianzani, M. U., *Biochim. Biophys. Acta,* 1954, v14, 514.

24. Hoagland, M. B., and Novelli, G. D., *J. Biol. Chem.,* 1954, v207, 767.

25. Brenner-Holzach, O., and Raaflaub, J., *Helv. Physiol. Acta,* 1954, v12, 242.

26. Ciaranfi, E., *Atti Soc. it. Pat.,* 1953, v3, 7.

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Determination of L-Glutamic Acid by Use of L-Glutamic Acid Decarboxylase from *Mycobacterium phlei.* (22266)

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Amino-acid decarboxylases of bacterial origin are now widely used for the quantitative estimation of certain amino-acids(1-7,9). Gale(1) suggested the use of *Clostridium welchii* SR-12 for the determination of glutamic acid. This organism releases CO_2 not only from L-glutamic acid, but from L-glutamine(3), and L-aspartic acid(5,6) as well. Additional tests and special conditions are necessary(3,5,6) to obtain separate values for each of these compounds. Glutamic acid decarboxylases obtained from different strains of *E. coli* were used by several investigators(2,7-9) for the assay of L-glutamic acid. In general, the *E. coli* preparations possessed in addition to L-glutamic acid decarboxylase, a glutaminase(7), or a L-lysine and L-arginine decarboxylases(2,8,9). Recently, Najjar and Fisher(9) reported that they were able to destroy the arginine and lysine decarboxylases without seriously affecting the L-glutamic acid decarboxylase.

In the present paper a description of the use of cell-free extracts from *Mycobacterium phlei* for the determination of L-glutamic acid will be given. Extracts from this organism decarboxylate quantitatively L-glutamic acid only and do not react with 18 other amino-acids tested. Furthermore, no glutaminase or glutamo-racemase activity could be detected under conditions optimal for the decarboxylase activity.

Methods. A strain of *Mycobacterium phlei* from the stock of this laboratory is

grown in Roux bottles on nutrient agar medium (peptone—1%, meat extract "Bovril" —0.3% • NaCl—0.50%, Na_2HPO_4 • $12H_2O$ —0.25%), to which 0.1% Tween 80 was added, and incubated for 3-4 days at 35°C. The total crop from 6 bottles is washed 3 times with saline, resuspended in 20 ml distilled water and then disintegrated in a 9 KC Raytheon sonic vibrator for 25 minutes. An alternative method for preparing the extracts by grinding the bacterial paste with alumina A-301, according to McIlwain(10) was also used and proved quite satisfactory. After disruption of the cells the debris are removed by a Servall centrifuge at 10,000 r.p.m. for 20 minutes and the clear and somewhat opalescent supernatant is dialyzed against running tap water in the cold-room for 13-15 hours. The protein content of the dialyzed solution is determined by Mehl's Biuret method(11).

Results. Measurements of glutamic acid decarboxylation. a). The Warburg manometric technic is used and from the amount of CO_2 evolved the quantity of glutamic acid that underwent decarboxylation can be calculated. Suitable controls containing no substrate and other controls containing substrate with KOH in the central well are run simultaneously with the complete system. All measurements are made at 37°C in citrate-phosphate buffer, pH 5.2, final concentration as indicated by McIlvaine(12), with air as the gas phase. The main compartment contains the enzyme and the buffer; the substrate is tipped in from the side-arm after equilibration for 20 minutes. For estimation of the reaction rate measurements are taken every 5-10 minutes. For quantitative estimation of glutamic acid readings are made until no changes in gas pressure are seen. The recovery of glutamic acid is 95-98% and owing to the low pH of the system there is hardly any retention of CO_2 by the liquid phase. b) Paper chromatography was used as an alternative method for the quantitative determination of the other product of decarboxylation of glutamic acid—γ-amino-butyric acid. Circular chromatography after Giri *et al.*(13) was employed; the solvent used was water-saturated phenol. The effect of pH on the activity of the enzyme is presented graphically in Fig. 1. The optimum activity is at pH 5.2-5.4, falling off rapidly at both sides of the pH curve, the slope being even steeper on the alkaline side.

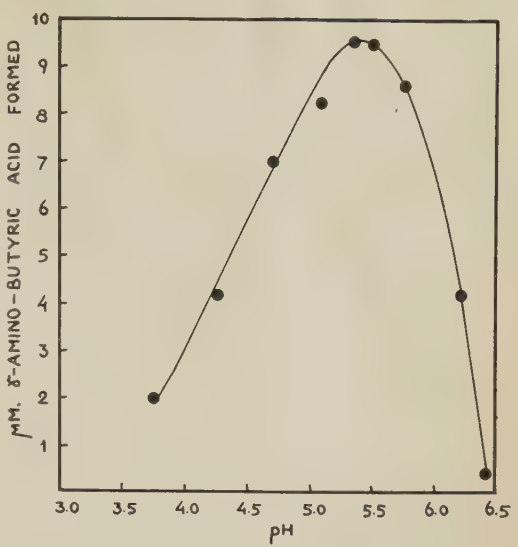

FIG. 1. The system contained: 10 μM L-glutamic acid, cell-free extract equivalent to 6.8 mg protein, McIlvaine's buffer, pH values as indicated, total volume—1 ml; time of incubation—2 hr at 37°C. The γ-amino butyric acid was determined by quantitative paper chromatography(13).

The influence of enzyme concentration on the reaction rate was studied and as is appar-

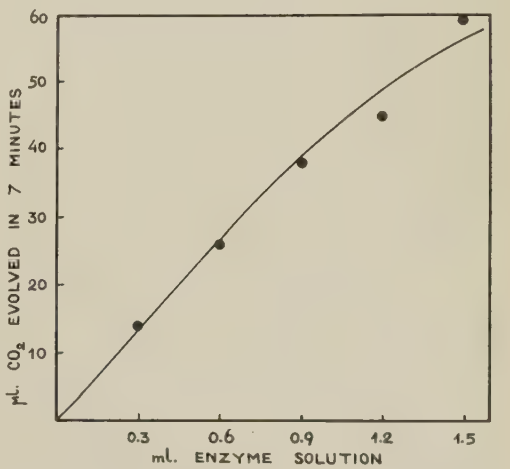

FIG. 2. The system contained: 10 μM L-glutamic acid, varying quantities of a cell-free extract (7.5 mg protein/ml), McIlvaine's buffer—pH 5.2; total volume—2.5 ml. Incubation at 37°C.

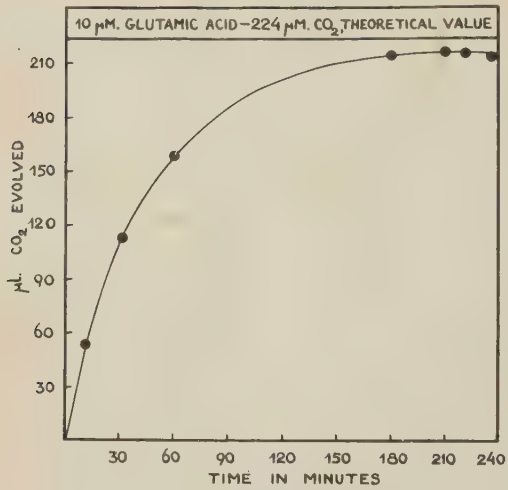

FIG. 3. The system contained: 10 μM L-glutamic acid, cell-free extract equivalent to 15 mg protein, McIlvaine's buffer—pH 5.2; total volume—2.5 ml. Incubated at 37°C.

ent from Fig. 2, activity is proportional to enzyme concentration as long as the enzyme is saturated with substrate. For the assay the enzyme is always taken in excess (15-20 mg protein per vessel). A typical experiment is illustrated in Fig. 3. Dialysis of the enzyme solution is not essential. However, when undialyzed extracts were used, the blanks showed considerably higher values. Only slight differences in activity (about 10%) were noticed when dialyzed and undialyzed extracts were compared. The enzyme preparation is quite stable; about ⅔ of activity were preserved after storage at 0°C for 4 months.

Specificity for L-glutamic acid. In repeated experiments with a series of amino-acids the cell-free extracts from *Mycobacterium phlei* did not decarboxylate any amino-acid other than L-glutamic acid. The amino-acids tested were: L-alanine, L-arginine, L-aspartic acid, D-glutamic acid, L-glutamine, glycine, DL-histidine, DL-hydroxyproline, DL-isoleucine, L-leucine, L-lysine, DL-methionine, DL-ornithine, DL-proline, DL-serine, DL-threonine, DL-tyrosine, L-valine. Furthermore, there is no appreciable change in decarboxylation rate of L-glutamic acid when D-glutamate, L-aspartate, L-glutamine, or L-asparagine are present. (Table I).

Partial purification of enzyme. A cell-free extract was prepared as already described. Ammonium sulphate was added gradually to obtain 33% saturation. The precipitate (fraction I) was centrifuged off and to the clear supernatant a further amount of ammonium sulphate was added until 50% saturation was obtained; centrifugation was then repeated and after removing the precipitate (fraction II) more ammonium sulphate was added to the supernatant until 70% saturation was reached. The precipitate (fraction III) was removed by centrifugation. All the manipulations were carried out in the cold-room. Table II shows the activities of the various fractions (after dialysis to remove the $(NH_4)_2SO_4$) as tested for glutamic acid decarboxylase. Fraction II (precipitate from 33-50% saturated $(NH_4)_2SO_4$ solution) contained over 50% of the original activity with a 3-4-fold purification.

Discussion. The enzyme preparation described in this paper has the advantage of being highly specific towards L-glutamic acid; none of the other 18 amino-acids tested is attacked by the enzyme. Closely related amino acids and amides (D-glutamic acid, L-glutamine, L-aspartic acid and L-asparagine) did not interfere with the activity of the glutamic decarboxylase. The lack of any measurable glutaminase activity enables direct estimation of L-glutamic acid in natural

TABLE I. Influence of Related Substances on Decarboxylation of L-Glutamic Acid.

Substrates (μM)	CO_2 evolved* (μM)	γ-amino butyric acid formed* (μM)
L-glutamic acid, 10	.60	.60
D-glutamic acid, 10		.0
L-glutamine, 10	.0	.0
L-aspartic acid, 10	.0	.0
L-glutamic acid, 10 + D-glutamic acid, 10		.58
L-glutamic acid, 10 + L glutamine, 10	.60	.58
L-glutamic acid, 10 + L-aspartic acid, 10		.62
L-glutamic acid, 10 + L-asparagine, 10		.62

* Values calculated/mg enzyme protein/hr.

The reaction mixture was set up in McIlvaine's buffer, pH 5.2, in a total volume of 1 ml, and incubated at 37°C; the γ-amino butyric acid formed was determined by quantitative paper chromatography (13).

TABLE II. Partial Purification of L-Glutamic Acid Decarboxylase with Ammonium Sulfate.

	Total activity (units)*	Total protein (mg)	Specific activity (units per mg protein)
Crude extract	216	1200	.18
F I .33 s†	46	760	.06
F II .50 s	113	180	.63
F III .70 s	5	40	.12
Supernatant at .70 s	0	213	.0

* 1 unit is defined as that amount of enzyme which will decarboxylate 1 μM of glutamic acid in 10 min.

† F = fraction; s = saturation.

The tests were performed with dialyzed enzyme samples; the γ-amino butyric acid formed was determined by quantitative paper chromatography.

products containing considerable amounts of glutamine. The cultivation of the bacteria and the preparation of the extract do not present any difficulties. It is possible to prepare a larger amount of extract and preserve it for a long period by cold-storage.

Summary. 1. A preparation from *Mycobacterium phlei* with decarboxylase activity towards L-glutamic acid only, was described. The preparation has no detectable decarboxylase activity towards other 18 amino-acids tested, and no glutaminase or glutamo-racemase activities. This enables direct estimation of L-glutamic acid in natural products. 2. The optimal pH range is between pH 5.2-5.4, with a sharp drop in activity at pH values of 4 and 6 respectively. 3. Some purification of the enzyme was achieved by fractionation with ammonium sulphate.

1. Gale, E. F., *Biochem. J.,* 1945, v39, 46.

2. Umbreit, W. W., and Gunsalus, T. C., *J. Biol. Chem.,* 1945, v159, 333.

3. Krebs, H. A., *Biochem. J.,* 1948, v43, 51.

4. ———, *ibid.,* 1950, v47, 605.

5. Meister, A., Sober, H. A., and Tice, S. V., *J. Biol. Chem.,* 1951, v189, 591.

6. ———, *ibid.,* 1951, v189, 577.

7. Ayengar, P., Roberts, E., and Ramasarma, B., *ibid.,* 1951, v193, 781.

8. Najjar, V. A., and Fisher, J., *Fed. Proc.,* 1952, v11, 264.

9. ———, *J. Biol. Chem.,* 1954, v206, 215.

10. McIlwain, H., *J. Gen. Microbiol.,* 1948, v2, 288.

11. Mehl, J. W., *J. Biol. Chem.,* 1945, v157, 173.

12. McIlvaine, T. C., *ibid.,* 1921, v49, 183.

13. Giri, K. V., Radhakrishnan, A. N., and Vaidyanathan, C. S., *Nature,* 1952, v170, 1025.

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Interrelationships of Glucose and Inorganic Phosphorus in Blood and Urine of Patients with Diabetes Mellitus.* (22267)

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Studies in relationships of inorganic phosphorus and glucose in blood, both in experimental animals and in man, have contributed to the development of present-day concepts concerning the role of phosphorus in metabolic processes(1). It has been established that marked lowering of blood glucose following administration of insulin is associated with simultaneous fall in phosphorus in blood and urine(2,3). A similar decline in inorganic phosphate also occurs following administration of glucose(4-6). The fall in serum phosphate has been shown to be related to active entry of glucose into the glycolytic cycle in muscle, rather than into the glycogeneic cycle in the liver(7). These observations have been extended and related to the action of insulin, epinephrine, and certain hypophyseal hormones(8). On the basis of these findings, the fall of serum inorganic phosphorus following intravenous administration of glucose has become an accepted criterion for peripheral utilization of glucose.

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TABLE I. Summary of Pertinent Data and Correlation Coefficients between Inorganic Phosphorus and Glucose.

Patient	Blood glucose, range, mg %	Plasma inorganic P, range, mg %	Urine glucose, range, g/24 hr	Urine inorganic P, range, g/24 hr	Days observed	Correlation coef. between blood glucose and plasma inorg. phosphorus	Correlation coef. between urine glucose and urine inorganic phosphorus
J.F.	36-468	3.0-5.2	.5- 90.8	.45- .90	33	−.73†	−.20
R.E.	90-211	2.8-3.5	.3- 11.9	.41-1.19	37	−.27	.21
F.H.	96-451	3.0-4.4	.8-140.0	.40-1.22	22	−.20	.58†
E.M., '53	87-368	3.6-4.7	5.1-116.0	.72-1.31	12	−.68*	.51
'52	60-367	1.8-3.7	—	—	32	−.62†	—
M.K.	53-442	3.4-5.0	—	—	36	−.26	—
J.M.	74-325	3.4-5.0	.4- 33.5	.43-1.42	24	−.15	.27
M.W.	50-331	2.4-4.4	.8- 42.7	.29- .79	32	−.69†	.37*
H.S.	46-356	1.9-4.2	—	—	31	−.15	—
M.A.	88-163	3.4-4.9	2.9- 29.3	.83-1.18	6	−.96†	.06
G.C.	145-473	1.8-4.4	—	—	23	−.28	—
G.M.	44-540	2.7-3.9	.2-144.9	.86-1.72	26	−.46*	.33
M.S.	44-369	2.9-5.0	.6- 83.5	.40-1.17	30	−.68†	.30
J.B.	30-244	2.2-5.0	—	—	33	−.43*	—
L.F.	75-353	2.6-5.0	1.0- 85.3	.42-1.26	42	−.44*	.18
S.D.	57-267	3.5-5.6	.7- 9.3	.78-1.20	11	−.72*	−.07

* $P < .05$. † $P < .01$.

Clinically, changes in serum inorganic phosphorus during intravenous glucose tolerance test have been used to differentiate diabetes mellitus from hyperglycemia of liver disease and other syndromes(9-12).

With these concepts in mind we set about to determine the relation of blood phosphorus to spontaneous and induced changes in blood sugar levels in patients with diabetes mellitus. The purpose of this paper is to report yet another relationship between plasma inorganic phosphorus and blood sugar which to our knowledge has not been described previously.

Procedure and methods. Phosphorus-glucose relationships were determined in 15 diabetic patients studied on the Metabolism Ward for prolonged periods. Ten patients had unstable diabetes; 4 were of the stable type(13). The group covered a wide range in age, duration of diabetes, and amount of exogenous insulin required. Throughout hospitalization, each patient received a chemically constant diet of identical foods and food values, the same menu being served every day. Data were obtained under optimal and suboptimal insulin regulation. Blood glucose and plasma or serum inorganic phosphorus were determined daily from the same sample of blood taken each morning before breakfast. Blood and urinary glucose were analyzed according to Somogyi's modification of the Shaffer-Hartman copper-iodometric titration method for "true" blood sugar(14). Plasma or serum inorganic phosphorus was determined either by modification of the method of Kitson and Mellon(15) or the method of Fiske and SubbaRow(16) and the urinary inorganic phosphorus by the method of Fiske and SubbaRow.

Results. A summary of the pertinent data is presented in Table I. It will be noted that there is a very wide range of blood glucose levels and, correspondingly, a fairly wide variation in plasma or serum inorganic phosphorus values. These 2 variables appeared to be related in a reciprocal manner, so that in general, low blood sugar levels tended to be associated with high plasma inorganic phosphorus levels and vice versa. In fact, a uniformly negative correlation between the 2 was found in all of the 15 patients studied (Table I). However, coefficients of correlation varied markedly from patient to patient. The degree of correlation did not appear to be dependent upon age, duration or type of diabetes, or amount of exogenous insulin required. Fig 1 illustrates the relationship between individual values for blood sugar and plasma inorganic phosphorus in a patient showing a fairly high coefficient of correlation.

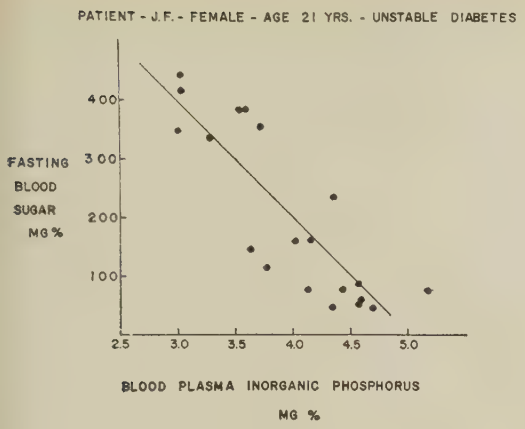

FIG. 1. Correlation between daily fasting blood sugar and plasma inorganic phosphorus in a patient with unstable diabetes mellitus.

In the one patient (E.M.) who was studied on 2 separate occasions, one year apart, the coefficients of correlation were found to be remarkably close, *i.e.*, –0.68 and –0.62 respectively. In contrast to the negative correlation between blood glucose and plasma inorganic phosphorus there appeared to be a slight tendency, if any, toward a low positive correlation between the 24-hour excretion of glucose and inorganic phosphorus in the urine.

Fig. 2 and 3 illustrate graphically the day-to-day levels of glucose and inorganic phosphorus in blood and urine and their interrelationships under different levels of insulin regulation in a patient with stable diabetes (M.W.) and in one with unstable diabetes (M.S.), respectively. It will be seen that in both patients, under optimal regulation, the spontaneous changes in blood sugar either above or below the normal base line appear to be associated with changes in plasma inorganic phosphorus in an inverse manner. On withdrawal of insulin (Fig. 2) or reduction of dosage to 50% or 60% of optimal (Fig. 3) the ensuing hyperglycemia is associated with a concomitant fall in plasma inorganic phosphorus. Note that the fall in inorganic phosphorus associated with the rise in fasting blood sugar in Patient M.W. occurred before the onset of the delayed glycosuria and was not associated with any appreciable change in the 24-hour excretion of inorganic phosphorus. Ketosis was absent in Patient M.W. In Patient M.S. ketonuria did not occur until several days of suboptimal insulin therapy had elapsed.

Discussion. The data presented demonstrate beyond all generally accepted statistical standards of reasonable doubt that, in the diabetics studied, fasting blood sugar and plasma phosphate level are negatively correlated. Whether or not this represents a causal relationship cannot be established by

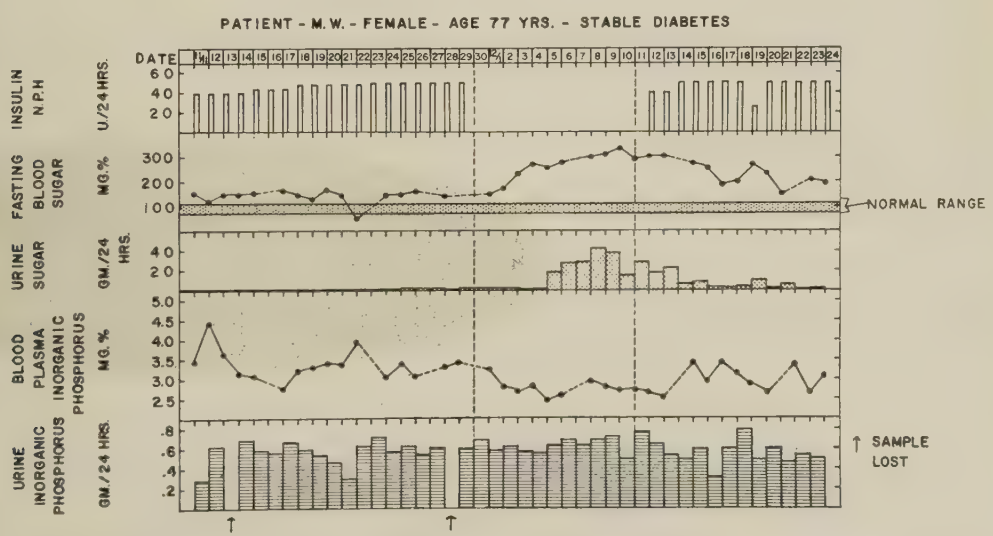

FIG. 2. Illustration of interrelationships of daily levels of glucose and inorganic phosphorus in blood and urine in a patient with diabetes mellitus under (a) optimal insulin regulation and (b) insulin withdrawal for an 11-day period.

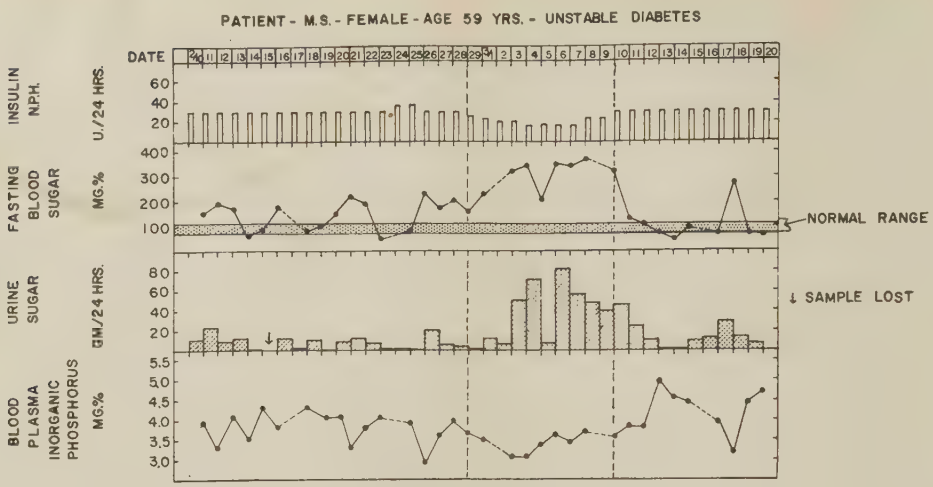

FIG. 3. Illustration of interrelationships of daily levels of inorganic phosphorus and daily levels of glucose in blood and urine in a patient with unstable diabetes under (a) optimal and (b) suboptimal insulin regulation.

this type of study. The consistency and reproducibility of the relationship in a given individual both with spontaneous changes in blood sugar and with changes induced by varying the dose of exogenous insulin would seem to favor a causal relationship. At any rate, the phenomena observed are of considerable interest since they are the reverse of those that had been anticipated in light of changes in serum phosphate with glucose utilization reported in short term experiments(2-12). If low blood sugar levels are taken as an index of increased utilization of glucose, one might expect low rather than high levels of phosphate. Conversely, decreased utilization of glucose as evidenced by hyperglycemia and glycosuria should lead to an accumulation of inorganic phosphorus in blood and urine. Indeed, elevation of both plasma and urinary phosphate does occur in diabetic acidosis; insulin therapy reduces these levels precipitously(17,18). In the present studies acidosis was absent. It should be pointed out that the present studies are not strictly analogous to the short term studies just alluded to. There is evidence that the short term change in phosphate caused by injection of glucose and insulin intravenously in diabetics is similar to that of normals(12). It is therefore possible that the phosphate-glucose relationships described reflect a different type of activity.

Can the observed phenomena be explained in part on a renal basis? Induction of hyperglycemia and glycosuria in normal animals (19) and man(20) has been shown to increase urinary excretion and clearance of phosphate while depressing tubular reabsorption of phosphate. In the present studies glycosuria does not appear to be an important factor in producing the phosphate changes since (1) the phosphate-glucose relationships occur in the absence of glycosuria and (2) phosphate and glucose excretion show at best only a very low positive correlation. This suggests that the observed phenomena reflect altered equilibria between phosphate stores and circulating phosphate rather than alterations in the renal mechanism governing phosphate excretion.

Summary. In a metabolic study of 15 patients with diabetes mellitus, a reciprocal relationship between fasting blood glucose and plasma or serum inorganic phosphorus was observed in all cases. In general, low levels of blood sugar tended to be associated with high levels of inorganic phosphorus and vice versa. Although a negative correlation was found in all patients, the coefficient of correlation varied markedly from patient to patient. There appeared to be a slight tendency, if any, toward a low positive correlation between the 24-hour excretion of glucose and inorganic phosphorus in the urine. The significance and possible mechanisms of the observed interrelationships are discussed.

We are indebted to Dr. S. Lee Crump, University of Rochester Medical Center, for statistical analyses in this paper.

1. McElroy, W. D., and Glass, B., Ed., *Phosphorus metabolism: a symposium on the role of phosphorus in the metabolism of plants and animals,* Baltimore, Johns Hopkins Press, 1951-52.

2. Harrop, G. A. J., and Benedict, E. M., PROC. SOC. EXP. BIOL. AND MED., 1923, v20, 430.

3. Wigglesworth, V. B., Woodrow, C. E., Smith, W., and Winter, L. B., *J. Physiol.,* 1923, v57, 447.

4. Fiske, C. H., *J. Biol. Chem.,* 1921, v49, 171.

5. Blatherwick, N. R., Bell, M., and Hill, E., *ibid.,* 1924, v61, 241.

6. Sokhey, S. S., and Allan, F. N., *Biochem. J.,* 1924, v18, 1170.

7. Pollack, H., Millet, R. F., Essex, H. E., Mann, F. C., and Bollman, D. L., *Am. J. Physiol.,* 1934, v110, 117.

8. Soskin, S., Levine, R., and Hechtor, O., *ibid.,* 1941, v134, 40.

9. Hartman, F. W., and Bolliger, A., *J.A.M.A.,* 1925, v85, 653.

10. Barrenscheen, H. K., Dolleschall, F., and Popper, L., *Biochem. Z.,* 1926, v117, 76.

11. Hartman, F., and Foster, D. P., *Am. J. Clin. Path.,* 1932, v2, 289.

12. Forsham, P. H., and Thorn, G. W., *Proc. Am. Diabetes Assn.,* 1949, v9, 101.

13. Izzo, J. L., *et al., J. Clin. Invest.,* 1950, v29, 1514.

14. Somogyi, M., *J. Biol. Chem.,* 1946, v160, 69.

15. Simonsen, D. G., Wertman, M., Westover, L. M., and Mehl, J. W., *ibid.,* 1946, v166, 747.

16. Fiske, C. H., and SubbaRow, Y., *ibid.,* 1925, v66, 375.

17. Guest, O. M., and Rapoport, S., *Am. J. Dis. Child.,* 1939, v58, 1072.

18. Franks, M., Berris, R. F., Kaplan, N. O., and Myers, G. B., *Arch. Int. Med.,* 1948, v81, 42.

19. Pitts, R. F., and Alexander, R. S., *Am. J. Physiol.,* 1944, v142, 648.

20. Levitan, B. A., and Stead, E., *J. Appl. Physiol.,* 1951, v4, 224.

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Reactions of Cyanocobalamin and Aquocobalamin with Proteins.* (22268)

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(Introduced by Earl A. Hewitt.)

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It now appears probable that absorption, transport, storage and function of cobalamins are accomplished by vitamin-protein complex formation. Although there is ample evidence that cobalamins are bound by proteinaceous substances present in gastric and duodenal secretions, blood serum, liver tissue, and milk there is relatively little information concerning the nature of bonds involved in the binding reactions. Cooley and co-workers(1) postulated that cobalamin-protein reactions may occur via cobalichrome formation analogous to the formation of ammonia and histidine cobalichromes. Van der Zant and Underkofler(2) presented evidence that suggests participation of both cobalt and substituted benzimidazole of cobalamins in cobalamin-gastric mucosal extract reaction. Absorption spectrum of purified cyanocobalamin-gastric protein complex, reported by Wijmenga and co-workers(3), indicates that cyano-group may still be present in the complex. Gregory and Holdsworth(4) reported the isolation of a similar cobalamin-protein complex from sow's milk and presented evidence indicating that the cyano-group is not displaced in the formation of this complex, and that the complex will not accept a second cyano-group. The latter authors made an extensive study of this binding reaction and concluded that tyrosyl residues of proteins but not sulfhydryl or amino groups, may participate. This investigation presents further

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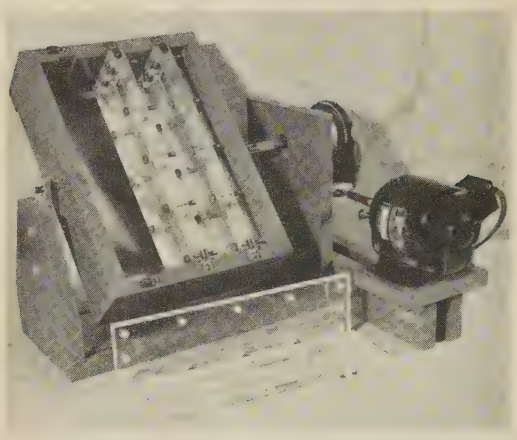

FIG. 1. Dialysis cells and rotation apparatus.

evidence concerning mode of linkage involved in cobalamin-protein reactions.

Dialysis method. Obvious inconsistencies in bacterial inhibition(5,6) and bacterial uptake(7) methods for differentiating free and bound cobalamins led to adoption of a dialytic procedure and radioisotope assay. Initial trials using dialysis bags and dialyzing to equilibrium or exhaustion, indicated that these methods were time-consuming and mechanically awkward. By standardizing the volume, membrane area and agitation a short-term or partial dialysis method was devised. Three identical Plexiglas dialysis cells were used. Each cell half contained a milled-out channel of about 30 ml capacity and a filling hole. The membrane, Visking dialysis tubing slit along one edge, was stretched between the two sections, separating and sealing the channels when cell halves were bolted together. Cells and rotation apparatus (3 r.p.m.) are shown in Fig. 1. In use, 25 ml of buffered protein-Co^{60}-labeled cobalamin mixture were dialyzed against equal volume of the same buffer for 4 hours in the dark at room temperature. Both solutions were then subjected to radio assay using a G-M liquid counting detector. Concentration of protein was adjusted to bind about half of the labeled cobalamin present. Using dialysis rate of free cobalamin, as determined by omitting protein from one of the 3 cells, the amount of cobalamin rendered non-dialyzable by a given amount of protein was calculated. Radioactive cobalamins were derived from a Co^{60}-labeled preparation (225 μc/mg cobalamin) obtained from Merck and Co. on AEC allocation. Cyanocobalamin (vit. B_{12}) was prepared by treatment of an aqueous solution of this preparation with an excess of NaCN followed by nitrogen aeration in the dark at pH 6.5. Aquocobalamin (vit. B_{12b}) was prepared by simultaneous illumination and nitrogen aeration of an aqueous solution of this preparation at pH 5.5. All subsequent operations using these preparations were carried out under conditions of minimum light. Dialysis rate of vit. B_{12} followed Fick's diffusion law, that is, directly proportional to concentration differential across membrane at constant temperature and membrane area, and was unaffected by buffer type or concentration, or pH over the range 2 to 10. Dialysis rate of vit. B_{12b} was less straightforward, being complicated by adsorption by the cellophane membrane, and perhaps dimerization, the effect being more prominent in media of low ionic strength. By using 0.2 M phosphate buffer reproducible dialysis of vit. B_{12b} was obtained at observed rate approaching that of vit. B_{12}.

Results. Using the partial dialysis method, it was found that many proteins bind vit. B_{12b} to a much greater extent than vit. B_{12}, as indicated in Table I. Although not hitherto

TABLE I. Binding of Vit. B_{12} and B_{12b} by Proteins.

Protein preparation*	Bound cobalamin, mμg/mg N†		
	B_{12}, pH 6.6	B_{12b}, pH 6.6	B_{12b}, pH 4.0
Whole hemolysed blood	0	3.8	—
Hemolysed red cells	0	3.0	—
Plasma	0	5.0	.7
Plasma, heat denatured‡	0	5.8	—
Albumin (Armour)	0	3.0	.7
Lysozyme, 2× crystallized	0	0	—
Lysozyme, alkali denatured§	0	3.7	—
Ovalbumin	0	3.7	—
Trypsin, 2× crystallized	0	2.5	.5
Pepsin, " "	0	5.1	—
Gastric mucosal extract	340	340	320

* Preparations contained 1 mg N/ml (0.013 mg N/ml for gastric mucosal extract) and 7.6 mμg cobalamin/ml in 0.2 M phosphate buffer. Blood preparations were of bovine origin.

† Sensitivity ca. 0.2 mμg cobalamin/mg N.

‡ Heated at 100°C for 5 min.

§ Incubated in 0.1 N NaOH at 37°C for 2 hr.

reported, this difference in extent of reaction between the two forms of vitamin can be expected as manifestation of cobalichrome formation. It was found that denaturation increased the vit. B_{12b} binding capacity of plasma and lysozyme. The binding capacity of 3 preparations was markedly decreased in acid media. Unreported spectrophotometric studies(8) indicated that vit. B_{12b}-histidine reaction is similarly depressed in acid media, as expected, and that histidine is the only amino acid giving evidence of cobalichrome formation in neutral solution. Similar compounds such as histamine, imidazole, carnosine, histidyl histidine and pyridine were found to form cobalichromes with vit. B_{12b}.

However, it cannot be inferred that benzimidazole is the only possible vit. B_{12b} binding site, for it was observed(8) that substances such as cellophane, filter paper, heparin, and nucleic acids (DNA and RNA) rendered nondialyzable considerable amounts of vit. B_{12b}. These substances did not bind measurable amounts of vit. B_{12}. The vit. B_{12b} bound by these substances and by the protein preparations was released by treatment with excess of cyanide in neutral solution.

The binding of as much as 5 mμg of vit. B_{12b}/mg of N by plasma is of interest for the physiological level is in the order of only 0.02 mμg of cobalamin/mg of N. Pitney, Beard and Van Loon have reported(9) that naturally occurring cobalamins are present in alpha and beta globulin components and that these components may also bind additional amounts of cobalamins, presumably vit. B_{12}, to a maximum of about 0.05 mμg/mg of N.

The position of bound labeled vit. B_{12} and B_{12b} in bovine serum was determined by subjecting to paper electrophoresis exhaustively dialyzed serum-cobalamin mixtures, and measuring radioactivity of the separate protein components(10). Serum used in this study was found to retain 4.3 mμg vit. B_{12b} or 0.092 mμg vit. B_{12}/mg of N, after exhaustive dialysis. Results presented in Table II indicate that all fractions of serum participated in binding of vit. B_{12b}, whereas only beta and gamma globulin components bound vit. B_{12}.

TABLE II. Position of Bound Co^{60}-Cobalamins in Bovine Serum Fractions Obtained by Paper Electrophoresis.

Preparation	Percent of activity			
	Albumin	α-	β-	γ-globulins
Serum + Co^{60}-B_{12b}	7	28	20	45
" " " -B_{12}	0	0	82	18

An entirely different situation was encountered in studying reaction of cobalamins with swine gastric mucosal extract. This extract consisted of water soluble, 50% alcohol insoluble, nondialyzable components of Ventriculin (Parke-Davis). As shown in Tables I and III, this preparation bound rather large and equal amounts of vit. B_{12} or B_{12b}, and binding of one form of vitamin was essentially blocked by prior excess of the other.

The effect of pH and excess cyanide on

TABLE III. Binding of Vit. B_{12} and B_{12b} by the Gastric Extract (GE).

Components*	Bound labeled cobalamin,† mμg/mg N
GE + Co^{60}-B_{12}	350
GE + excess B_{12b} + Co^{60}-B_{12}‡	0
GE + Co^{60} B_{12b}	350
GE + excess B_{12} + Co^{60}-B_{12b}‡	15

* Preparations contained 0.013 mg N/ml, 7.6 mμg labeled cobalamin/ml, and 2 μg nonlabeled cobalamin/ml, in 0.2 M phosphate buffer of pH 6.6.

† Sensitivity ca. 10 mμg cobalamin/mg N.

‡ Gastric extract and nonlabeled cobalamin allowed to react before adding labeled cobalamin.

TABLE IV. Effect of Hydrogen Ion Concentration and Excess Cyanide on Binding of Vitamin B_{12} by Gastric Extract.

pH*	Bound vit. B_{12}, mμg/mg N	
	No CN^-	Excess CN^-
2	320	—
4	320	—
6	330	—
6.6	340	340
8	320	—
10	330	250
10	340	250†
10	330	200‡
11	220	80
12	90	0

* Preparations contained 0.013 mg N/ml and 7.6 mμg Co^{60}-B_{12}/ml in 0.2 M phosphate buffer.

† Gastric extract and B_{12} allowed to react before adding cyanide. In all others, cyanide and B_{12} mixed before adding gastric extract.

‡ Contained 0.4 mg NaCN per ml. All others contained 0.1 mg NaCN/ml.

TABLE V. Fate of Cyano-Group of Vit. B_{12} in the Vit. B_{12}-Gastric Extract Reaction.

Treatment	Gastric extract	H_2O control
CN^- removed initially by N_2 aeration		
In dark, mμg	86	—
Illuminated, mμg	15	—
Idem	8	5
B_{12} added in dark, μg	4.5	4.5
(CN^- equivalent, mμg)	(87)	(87)
CN^- recovered by N_2 aeration		
In dark, mμg	14	12
Illuminated, mμg	79	83
B_{12} bound,* μg	3.4	—
(CN^- equivalent, mμg)	(66)	—

* Determined by the partial dialysis method.

binding of vit. B_{12} by this extract is shown in Table IV. The reaction was essentially unaffected by pH changes from pH 2 to 10 but was inhibited in more basic solution. Excess cyanide had no effect in neutral solution, but exerted a progressive inhibition above pH 10. The order of addition of excess cyanide and labeled vit. B_{12} to gastric extract did not affect the magnitude of inhibition. Excess cyanide did not affect the dialysis rate of free cobalamin. These data suggest that cobalamin is bound to this protein through the position sought by the second cyano-group.

That the cyano-group of vit. B_{12} is not displaced by the binding reaction is indicated by data presented in Table V. The cyanide method of Boxer and Rickards(11) was used. Gastric mucosal extract was first freed of cyanide by nitrogen aeration at pH 5. Labeled vit. B_{12} was then added and cyanide again determined, first after aeration in the dark, then while being illuminated. The amount of vit. B_{12} which had actually been bound by gastric extract was subsequently determined by partial dialysis. It appears that the first cyano-group is not displaced by the binding reaction, but remains in a photolabile position, presumably still with the cobalamin structure.

The effect of several treatments on vit. B_{12}-gastric mucosal extract reaction, as studied by partial dialysis, is shown in Table VI. The reaction was not affected by type or concentration of buffer. The effect of heat was similar to that reported by others using bacterial assay methods(2,12,13) and indicates that the complex is as heat-labile as the unreacted binding substance at pH 6.6. The report that cobalt-benzimidazole mixtures inhibit the reaction(2) could not be confirmed by this method. The suggestion that protein sulfhydryl groups may be involved in binding was shown to be unlikely, for the reaction was unaffected by excess para-chloromercuribenzoate. A cobalt porphyrin, reported to promote growth of vit. B_{12}-requiring microorganisms(14) was found to have no marked effect on the reaction.

Discussion. The marked reactivity of vit. B_{12b} suggests that suitable precautions should be taken against photoconversion when attempting to study vit. B_{12}-protein reactions. For example, heparin and nucleic acids were found to bind vit. B_{12b}, and not vit. B_{12} as had been reported(15). The binding or adsorption of vit. B_{12b}, by substances such as cellophane and filter paper point up difficulties involved in ascribing a physiological function to substances showing vit. B_{12b}-binding activity. Data presented in Tables I and

TABLE VI. Effect of Various Treatments on Binding of Vit. B_{12} by the Gastric Extract.

Treatment		Bound vit. B_{12}, mμg/mg N
Effect of buffer at pH 6.6		
No buffer		350
.1 M phosphate		320
.2 "		330
.4 "		330
.2 M acetate		340
Heating the gastric extract		
60°C, 1 hr		330
100°C, "		180
Heating the B_{12}-gastric complex		
60°C, 1 hr		330
100°C, "		180
Reagents	Molar excess*	
$CoCl_2$	2×10^6	320
Benzimidazole	3×10^5	330
" , $CoCl_2$	" , 2×10^6	320
" , " , heat†	" "	270
p-Chloromercuribenzoate	1×10^4	340
Cobalt-hematoporphyrin	1×10^5	310

* Relative to original B_{12} concentration, 7.6 mμg per ml or 5.6×10^{-9} M.

† Heated at 100°C for 10 min. prior to addition of B_{12}. Gastric extract alone given same heat treatment bound 290 mμg B_{12}/mg N.

II suggest that binding of vit. B_{12b} by proteins, possibly via a cobalichrome linkage with histidine residues, is a rather general reaction involving little structural specificity of the proteins involved, and may therefore be of little physiological significance.

The limited study of the cobalamin-gastric mucosal extract reaction reported here is in general agreement with the more extensive study of Gregory and Holdsworth(4) who used ultrafiltration to study binding of vit. B_{12} by an isolated milk protein. However, there appears to be some discrepancy concerning the effect of excess cyanide on the reaction or reaction product. Gregory and Holdsworth observed no changes in absorption spectrum of cyanocobalamin-milk protein complex in presence of excess cyanide at pH values as high as pH 11. The data presented here indicate that dicyanocobalamin, in presence of excess cyanide, does not react completely with gastric extract, that excess cyanide dissociates pre-formed cyanocobalamin-gastric extract complex, and that these reactions are dependent upon cyanide concentration and pH. These results may represent a difference in nature of the binding reaction by the 2 preparations, or a difference in molar excess of cyanide used in the two situations. Although there have been reports (16,17) that cobalamin-peptide complexes are split by cyanide in neutral solution, the possibility exists that these are cobalichrome type structures and thus more susceptible to cyanolysis.

Summary. 1. A short-term dialysis procedure has been developed for studying cobalamin-protein reactions. 2. Aquocobalamin (vit. B_{12b}) was much more reactive than cyanocobalamin, being bound in rather large amounts by several protein preparations of diverse origin. In several instances binding was increased by denaturation and decreased in acid media. It is suggested that this type of binding may occur via cobalichrome formation. 3. A gastric mucosal extract bound large and equal amounts of aquocobalamin and cyanocobalamin, apparently at the same binding site. Excess cyanide has no effect on the cyanocobalamin-gastric extract reaction in neutral media, but exhibits an inhibiting effect at high pH levels. In this reaction the cyano-group of cyanocobalamin is not released, but remains in a photolabile position. It is suggested that the cobalt atom participates in the reaction and that it is the position occupied by the substituted benzimidazole moiety that is involved.

1. Cooley, G., Ellis, B., Petro, V., Beaven, G. H., Holiday, E. R., and Johnson, E. A., *J. Pharm. and Pharmacol.,* 1951, v3, 271.

2. van der Zant, E. S., and Underkofler, L. A., *Proc. Iowa Acad. Sci.,* 1953, v60, 325.

3. Wijmenga, H. G., Thompson, K. W., Stern, K. G., and O'Connell, D. J., *Biochim. et Biophys. Acta,* 1954, v13, 144.

4. Gregory, M. E., and Holdsworth, E. S., *Biochem. J.,* 1955, v59, 335.

5. Bird, O. D., and Hoevet, B., *J. Biol. Chem.,* 1951, v190, 181.

6. Prusoff, W. H., Welch, A. D., Heinle, R. W., and Meacham, G. C., *Blood,* 1953, v8, 491.

7. Davis, R. L., and Chow, B. F., *Fed. Proc.,* 1953, v12, 440.

8. Bauriedel, W. R., Ph.D. Dissertation, Dept. of Chemistry, Iowa State College, 1954.

9. Pitney, W. R., Beard, M. F., and Van Loon, E. J., *J. Biol. Chem.,* 1954, v207, 143.

10. Schilling, R. F., and Deiss, W. P., PROC. SOC. EXP. BIOL. AND MED., 1953, v83, 506.

11. Boxer, G. E., and Rickards, J. C., *Arch. Biochem.,* 1951, v30, 372.

12. Bethell, F. H., Swendseid, M. E., Miller, S., and Citron-Rivera, A. A., *Ann. Int. Med.,* 1951, v35, 518.

13. Shaw, G. E., *Biochem. J.,* 1950, v47, xxxv.

14. Banhidi, Z. G., Sjostrom, A. G. M., and Paul, K. G., *Exp. Cell Research,* 1952, v3, 489.

15. Chow, B. F., and Davis, R. L., *Recent Adv. in Nutr. Res.,* 1952, v5, 17.

16. Wijmenga, H. G., Veer, W. L. C., and Lens, J., *Biochim. et Biophys. Acta,* 1950, v6, 229.

17. Hausmann, K., and Mulli, K., *Lancet,* 1952, v262, 185.

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Pituitary Stimulating Substance in Brain Blood of Hypophysectomized Rat Following Electric Shock "Stress".*† (22269)

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Release of ACTH following non-specific physiological stimuli is thought to involve the hypothalamus. However, the role of the hypothalamus in mobilization of ACTH is unclear. Following the ingenious experimental work of Harris and his co-workers (1-3), the concept of a hypothalamic humoral mediator in release of ACTH has gained wide support. The work of Harris *et al.*, Hume(4) and others(5-6), supports the thesis that areas in the posterior hypothalamus contain centers responsible for ACTH release. McCann and Sydnor(7) emphasize importance of anterior hypothalamus and suggest that pitressin, synthesized in this area(8), stimulates ACTH production following stress. The possible role of pitressin in stimulating release of ACTH has been emphasized by our work(9). However, Saffran *et al.*(10) reported that a contaminant of pitressin, but not pitressin itself, will cause ACTH release *in vitro*.

A minority of investigators, chiefly Vazquez-Lopez(11), believe that nerve fibers coursing in the hypothalamic-hypophyseal tract may serve to increase ACTH release by the anterior pituitary. To determine if a humoral substance is released from the hypothalamus following stress, the following experiment was devised: Venous brain blood of stressed hypophysectomized rat (donor animal) was administered into recipient animal in which endogenous mobilization of ACTH was impaired by electrolytic lesions placed in the hypothalamus (Fig. 1).

Methods. Hypophysectomy: Adult rats of the Addis-Slonaker strain were hypophysectomized and not used until at least 3 weeks later. The effect of ether anesthesia on eosinophils of several of these animals was determined. After each experiment, the sella was inspected with dissecting microscope for pituitary remnants. If found, the experiment was discarded. *Hypothalamic lesions*: Electrolytic lesions were placed in the hypothalamus by the Horsley-Clark stereotaxic apparatus as modified for the rat by Krieg(12). An indifferent electrode was placed in the rectum. The lesion electrode consisted of #21 gauge hollow tubing into which an entomological pin‡ was soldered at one end. The electrode was insulated with Formvar enamel§ except for 1 mm at tip of protruding pin. A current of 3 ma maintained for 15-20 seconds was used. Bilateral lesion sites were placed 4-7 mm anterior to intra-aural plane, 1 mm lateral and withdrawn 0.5-1 mm from base of skull. This location was generally within 1-2 mm of Bregma. Thirty to sixty % of the animals died within 2 days after the lesion. All lesioned rats surviving 3 weeks or more were subjected to ether anesthesia, found by Sydnor *et al.*(13) and Sayers *et al.*(14) to produce a stress reaction in rats as measured by increase in serum ACTH. Changes in level of circulating eosinophils were determined. Any rat having a decrease in eosinophils $>$ 20% was discarded as this indicated ability of lesioned rat to mobilize ACTH following stress was undisturbed. About 70% of lesioned rats were thus discarded. The remaining rats were used for experimental purposes. In addition, intact and adrenalectomized rats were exposed to ether and changes in eosinophils determined.

Experimental Procedure. Electric shock

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‡ No. A-80; Size 1—Clay Adams Co., New York.

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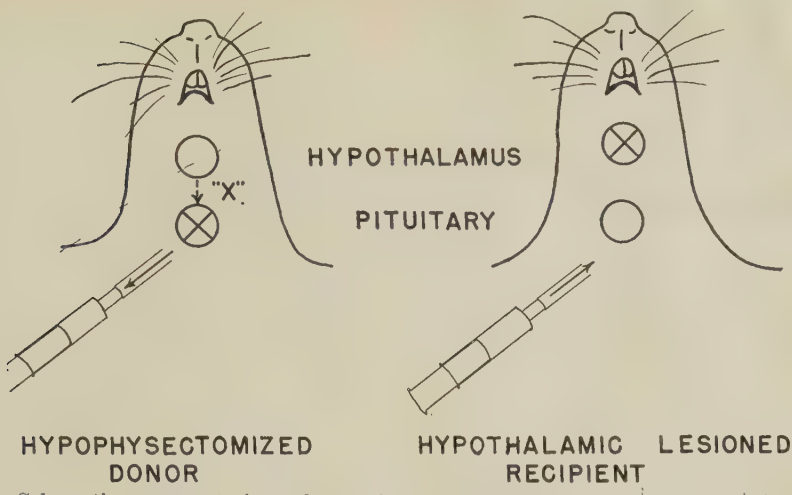

FIG. 1. Schematic representation of experimental design. The hypophysectomized rat is shocked with electricity and its venous brain blood collected in a syringe. This blood is injected intra-arterially into the hypothalamic lesioned rat. The dotted arrow represents a presumed secretion of the "Hypothalamic Hormone(s)" in response to the shock. Circles with × through them indicate removal of the designated areas.

stress was selected, for this could be controlled as to duration and intensity. An Ene-volt transformer¶ was employed. One lead was connected to each front foot and a current of approximately 24 volts administered at 3 per second for 2 minutes; 15 seconds on—5 seconds off. This constituted a severe stress, for the rats would struggle and convulse.

The experimental design is shown schematically in Fig. 1. At 9:00 A.M. a hypophysectomized donor rat was anesthetized with ether. The posterior facial vein (PF vein)** on one side was cannulated peripherally. The opposite PF vein was isolated and 0.2 cc heparin injected intravenously. The rat was maintained under light anesthesia for 60-90 minutes, then ether was withdrawn. Now the lesioned rat was anesthetized with ether and a blood sample immediately taken from free-flowing tail blood for eosinophil determinations. One carotid artery was cannulated peripherally; electric shock stress was then administered to the hypophysectomized donor rat. The isolated PF vein was ligated and about 2 cc of blood was withdrawn, during a 2-3 minute period, from the cannula in the contralateral PF vein. This blood was immediately injected into the lesioned recipient by means of the carotid cannula. Generally <15 minutes elapsed between anesthetizing the lesioned rat and injection of blood from the hypophysectomized rat. Eosinophils in the lesioned rat were then counted 30 minutes and 4 hours after injection of brain blood from the hypophysectomized rat. They were stained with propylene glycol-phloxine solution(15) and plated on a Fuchs-Rosenthal counting chamber. Four chambers were counted under low power. There was no evidence of blood incompatibility as determined by mixing 2 drops of blood from both donor and recipient animals.

Controls. Hypophysectomized rats were anesthetized with ether and the posterior facial vein was cannulated as described above. After 90 minutes, blood was withdrawn into the cannula and injected into the carotid artery of the same lesioned rats, which had received, or were subsequently to receive blood from "stressed"†† hypophysectomized rats.

¶ Correl & Correl, Chicago Heights, Ill.

** Dr. H. Hoagland(16), has shown that 70-80% of venous brain blood of the rat leaves the head via this vein.

†† Hypophysectomized rats shocked with electricity are hereafter referred to as "stressed" hypophysectomized rats.

TABLE I. Changes in Circulating Eosinophils in Rats 4 Hr after Exposure to Ether.

	(10) Normal	(8) Lesioned	(6) Hypophysectomized	(6) Adrenalectomized
% change in eosinophils	—58*	—11, —10, —20, —15, +13	+8, +43	—16
	—41	—20, —22	—15	— 3
	—35	+83	—25	+17
	—55	—19	—13, —15, +9	— 7
	—69	+18	—22	— 8
	—50	+16	— 8	—10
	—43	+ 7		
	—53	+14		
	—38			
	—55			

* (—) % decrease; (+) % increase.

Injection of blood from "unstressed"‡‡ hypophysectomized rats occurred either a week before or after injection of brain blood from "stressed" hypophysectomized rats. Brain blood from "stressed" hypophysectomized rats was injected into adrenalectomized rats and hypophysectomized rats prepared as indicated above for the lesioned rat.

Results. Effect of ether stress on circulating eosinophils. Eosinophil changes occurring in 10 normal rats following exposure to ether are shown in Table I. A marked decrease occurred in each animal. This eosinopenia did not develop in 6 hypophysectomized rats exposed to ether, nor in 6 adrenalectomized rats similarly treated. This indicates that eosinopenia, following exposure to ether anesthesia, depends upon an intact pituitary-adrenal axis. In 8 lesioned rats anesthetized with ether eosinopenia also did not occur (Table I). Thus, it appears that a functional hypothalamic-pituitary-adrenal axis is necessary for the eosinopenic effect of ether. Several rats cited above were tested more than once for their response to ether.

Injection of brain blood from "stressed" and "unstressed" hypophysectomized rats into lesioned rats. The 8 lesioned rats which indicated no eosinopenia, following ether (Table I), were injected with brain blood from "stressed" and "unstressed" hypophysectomized rats. Changes in eosinophils are shown in Fig. 2. Lesioned rat #1 received blood from 2 different "stressed" hypophysectomized rats twice during 2 weeks, while the other lesioned rats received one injection of brain blood from a "stressed" hypophysectomized rat, and one injection of brain blood from an "unstressed" hypophysectomized rat. Thus, each rat served as its own control. The decrease in eosinophils in lesioned rats following injection of brain blood from "stressed" hypophysectomized rats was 65-90%. Following injection of brain blood from unstressed hypophysectomized rats into these same lesioned rats, the decrease was generally 25-35%; in one animal, eosinophils actually increased. In each case, regardless of whether the lesioned rat received "stressed" or "unstressed" blood, the 30 minute count was higher than the initial count.

Injection of brain blood from "stressed" hypophysectomized rats into adrenalectomized rats and hypophysectomized rats. This procedure was undertaken to determine whether eosinopenia following injection of brain blood from "stressed" hypophysectomized rats, was mediated by the adrenal-pituitary axis. In 4 adrenalectomized rats injection of brain blood from "stressed" hypophysectomized rats caused a decrease in eosinophils of only 15%, 1%, 19% and 11%. In 4 hypophysectomized rats into which brain blood from "stressed" hypophysectomized rats was injected, there was a decrease in eosinophils of 26%, + 27%, 23% and 4%.

Location of lesion sites. The brain was fixed in 10% formalin and several weeks later, sectioned through the hypothalamic area and stained in hematoxylin and eosin. Lesions were of massive nature, as intended, for we desired to block mobilization of ACTH without regard to minute hypothalamic areas. In 4 rats in which lesion sites were determined, the following areas were involved:§§ Rat #1 posterior hypothalamus including mammillary bodies, tuber cinereum and mammilothalamic tract. Rat #2 posterior hypothalamus including mammillary bodies, pitui-

‡‡ Hypophysectomized rats not shocked with electricity are referred to as "unstressed" hypophysectomized rats.

§§ We wish to thank Dr. John D. Green for localizing the lesion sites.

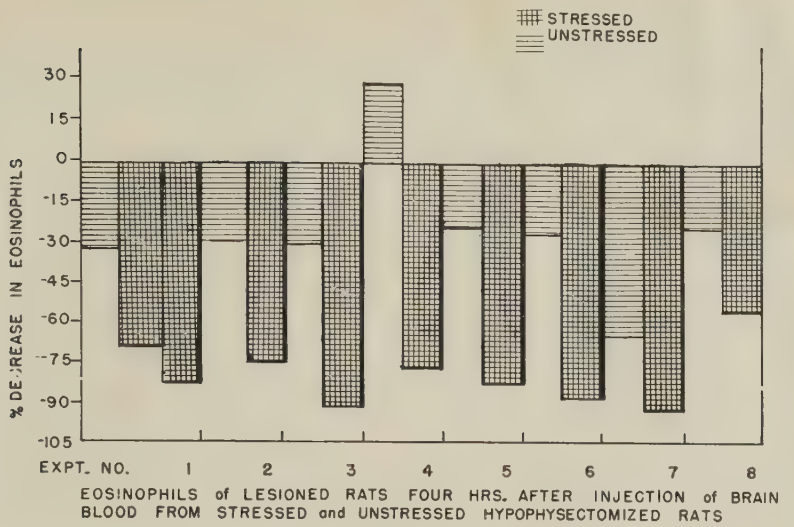

FIG. 2.

tary stalk, supraoptic and ventral hypothalamus. Rat #3 ventral hypothalamus including tuber cinereum; ventromedial nucleus and dorsomedial nucleus. Rat #4 posterior hypothalamus including tuber cinereum and mammillary bodies.

The location of massive lesions which blocked ACTH release, as measured by eosinopenia, were in the posterior and ventral hypothalamus and included mammillary bodies and tuber cinereum. McCann and Sydnor (7) observed that only anterior hypothalamic lesions, which destroyed the median eminence, blocked adrenal ascorbic acid depletion following laparotomy and subcutaneous epinephrine. Our lesion sites are in general agreement with Harris(1-3), Hume(4), Slusher & Roberts(6), and Porter(5), who similarly found areas in posterior hypothalamus responsible for ACTH mobilization. None of our lesioned rats demonstrated marked diabetes insipidus although daily water consumption records exceeded that of normal controls.

Discussion. Our data indicate that venous brain blood of "stressed" hypophysectomized rats will induce eosinopenia when injected into rats bearing hypothalamic lesions. This eosinopenia depends upon presence of intact pituitary-adrenal axis in recipient animal. Eosinopenia did not occur when brain blood from "unstressed" hypophysectomized rats was injected into these same lesioned rats. The assumption therefore appears justified that following "stress," brain blood of hypophysectomized animal contains a pituitary stimulating substance(s). These observations are compatible with the hypothesis of hypothalamic humoral control over ACTH secretion, and point to hypothalamus as the most probable site of hypophysiotropic principle demonstrated here. The demonstration that eosinopenia occurs only if pituitary and adrenal glands are present in recipient animal, further suggests that brain blood from "stressed" hypophysectomized rats exerts an eosinopenic effect via pituitary-adrenal axis, and has little, if any, direct effect upon level of circulating eosinophils.

The level of circulating eosinophils has been used by many workers investigating functional integrity of the hypothalamic-pituitary-adrenal axis(1,4,5). In some cases, however, eosinopenia may develop in adrenalectomized animals(17-18), following laparotomy and other severe stresses, although most investigators have found that this is not the case. We did not notice eosinopenia in adrenalectomized or hypophysectomized rats exposed to ether stress and minor neck surgery, nor did it occur in our rats with hypothalamic lesions. Thus under our conditions, eosinopenia depends upon intact hypothala-

mic-pituitary-adrenal axis.

Summary. Brain blood from "stressed" and "unstressed" hypophysectomized rats was injected into rats bearing hypothalamic lesions, and also into hypophysectomized and adrenalectomized rats. When brain blood from stressed hypophysectomized rats was injected into lesioned rats, a marked eosinopenia occurred; this did not occur when unstressed brain blood was injected into those same lesioned rats. This eosinopenia depended upon the presence of an intact pituitary-adrenal axis.

1. Harris, G. W., *Physiol. Rev.,* 1948, v28, 139.

2. Green, J. D., and Harris, G. W., *J. Endocrinology,* 1947, v51, 136.

3. deGroot, J., and Harris, G. W., *J. Physiol.,* 1950, v111, 335.

4. Hume, D. M., *Ann. Surgery,* 1953, v138, 548.

5. Porter, R. W., *Am. J. Physiol.,* 1953, v172, 515.

6. Slusher, M. A., and Roberts, S., *Endocrinology,* 1954, v55, 245.

7. McCann, S. M., and Sydnor, K. L., PROC. SOC. EXP. BIOL. AND MED., 1954, v87, 369.

8. Leveque, T. F., and Scharrer, E., *Endocrinology,* 1953, v52, 436.

9. Sobel, H., Levy, R. S., Marmorston, J., Schapiro, S., and Rosenfeld, S., PROC. SOC. EXP. BIOL. AND MED., 1955, v89, 110.

10. Saffran, M., and Schally, A. V., *Canad. J. Biochem. and Physiol.,* 1955, v15, 840.

11. Vazquez-Lopez, E., *J. Endocrinology,* 1949-1950, v6, 158.

12. Krieg, W. J. S., *Quart. Bull. Northwestern Univ. Med. School,* 1946, v20, 199.

13. Sydnor, K. L., and Sayers, G., *Endocrinology,* 1954, v55, 621.

14. Sayers, G., and Burke, R., *J. Clin. Endocrin. and Metab.,* 1955, v15, 840.

15. Henneman, P. H., Wexler, H., and Westenhaver, M. M., *J. Lab. and Clin. Med.,* 1949, v34, 1017.

16. Hoagland, H., *Recent Prog. Hormone Research,* 1954, v10, 29.

17. Dumm, M. E., and Ralli, E. P., *Endocrinology,* 1954, v54, 71.

18. Swingle, W. W., Eisler, M., Ben, M., Maxwell, R., Baker, C., and LeBrie, J., *Am. J. Physiol.,* 1954, v178, 341.

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Effects of Vitamin E, N,N^1-Diphenyl-Para-Phenylene Diamine, and Fish Liver Oil on Reproduction in Turkeys.*† (22270)

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A deficiency of vit. E has recently been reported in turkey breeder hens fed practical rations(1,2). The lenses of the eyes of embryos from the eggs laid by hens fed diets deficient in vit. E were cloudy in the central portion(3). In vit. E studies with chicks, Singsen *et al.*(4) found that the unsaturated fat level of the diet markedly influenced the development of encephalomalacia and that the antioxidant N, N^1 diphenyl-para-phenylene diamine (DPPD) was about as effective as vit. E in preventing the disease. The effects of DPPD, fish liver oil and alpha tocopheryl acetate on the reproductive performance of turkeys are reported herein.

Procedure. Broad Breasted Bronze turkey hens were distributed into 12 pens of 11 hens each. These birds had been fed a practical diet during the growing period and were reared on range. A basal diet of the following percentage composition was used: 66 ground corn, 18 soybean oil meal, 2.5 dehydrated alfalfa, 3 herring fish meal, 3 dried whey, 3.7 limestone, 3.5 steamed bone meal, 0.3 iodized salt, 0.0125 manganese sulfate and a vitamin mix supplying 680 I.C.U. vit. D, 1135 I.U. vit. A, 1 mg riboflavin, 6.2 mg pantothenic acid and 2.5 mg vit. B_{12} per lb of

* The authors wish to thank Mr. Hugh A. Risley, Distillation Products Industries, Rochester, N. Y., for determining tocopherol content of eggs in this study. The alpha tocopheryl acetate was supplied by Distillation Products Industries. The DPPD was supplied by B. F. Goodrich Chemical Co.

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TABLE I. Effect of Vit. E, Fish Liver Oil and DPPD on Reproductive Performance of Broad Breasted Bronze Turkeys and upon Tocopherol Content of the Yolks.

Supplement to basal	Pen No.	% fertility	% embryonic mortality		Pips	% hatch. fertile eggs	Total tocopherol, μg/g yolk
			1-24 days	25-28 days			
None	33	56.9	7.8	14.3	7.4	70.6	32.2
	39	75.7	8.7	12.7	12.7	62.2	31.3
	Avg	66.5	8.3	13.4	10.3	66.0	31.8
Vit. E	36	85.2	4.7	8.0	13.7	74.6	74.2
	41	78.5	5.8	7.9	7.2	79.5	71.2
	Avg	81.8	5.2	7.9	9.9	77.1	72.7
Pollack liver oil (PLO)	35	67.2	15.3	44.5	9.6	30.6	17.1
	37	52.2	7.1	36.3	8.8	47.8	17.8
	Avg	58.6	11.5	41.2	9.2	38.6	17.4
PLO + Vit. E	34	75.8	9.8	7.5	5.6	77.0	27.1
	42	80.8	5.4	9.0	9.7	76.0	16.0
	Avg	77.9	7.7	8.2	7.5	76.5	23.0
PLO + DPPD	31	73.7	7.3	13.7	7.7	71.4	16.5
	38	72.8	3.3	13.3	7.2	76.3	19.7
	Avg	73.2	5.1	13.5	7.4	74.0	18.0
PLO + DPPD + Vit. E	32	68.2	3.9	7.9	6.2	82.0	24.6
	40	70.2	9.3	6.9	5.8	78.0	23.7
	Avg	69.2	7.2	7.2	6.0	79.5	24.3

feed. Duplicate lots were fed the basal diet and the basal diet supplemented with 20 I. U./lb alpha tocopheryl acetate, 2% pollack liver oil, 2% pollack liver oil plus 20 I.U./lb alpha tocopheryl acetate, 2% pollack liver oil plus 0.025% DPPD and 2% pollack liver oil plus 20 I.U./lb alpha tocopheryl acetate. During the first 9 weeks of the experiment, Broad Breasted Bronze toms were maintained in a separate pen. Semen was collected from these toms and pooled for artificial insemination of the hens. During the last 12 weeks of the experiment, one tom was placed in each pen and the toms were rotated weekly. All eggs laid were stored at approximately 55°F and incubated every 2 weeks. The eggs were candled on the 7th and 24th day of incubation to determine infertile eggs and eggs containing dead embryos. The experiment was conducted for a period of 21 weeks. During the latter part of the experiment, a sample of 6 eggs from each pen was analyzed for total tocopherol content.

Results. The results of this experiment are presented in Table I. The data on fertility are based only on eggs laid by the hens after they were naturally mated. These results suggest that natural diets such as used may be deficient in vit. E to the extent of causing reduced fertility of eggs. The addition of alpha tocopherol acetate to both the unsupplemented basal diet and to the basal diet containing 2% fish liver oil increased the percentage of fertile eggs. In results with turkeys reported by other investigators(1,2) fertility was not affected by a vit. E deficiency. Singsen *et al.*(5), however, obtained evidence suggesting an improvement in fertility in chickens by addition of vit. E to the breeder ration. It should be mentioned that fertility in the experiment being reported was determined only by candling. It is possible that some of the eggs classified as infertile may have contained embryos that died at a very early stage.

Addition of vit. E to the basal diet increased hatchability of fertile eggs. When 2% fish oil was added to the basal diet, hatchability of fertile eggs decreased from 66 to 39%. The addition of vit. E or DPPD or a combination of the two in the presence of fish oil markedly improved hatchability. These results show that the presence of an unsaturated oil in the diet greatly accentuated the deficiency of vit. E. The results also show that both vit. E and DPPD act to

counteract the deleterious effect of unsaturated oil on hatchability. An examination of the data on time of embryonic mortality shows that there was very little difference in the percentage of embryos dying from 1 to 24 days among the various treatments. In the case of embryos dying during the 25-to-28-day period, however, there was a marked difference. When fish liver oil was added to the basal diet embryonic mortality during this period greatly increased. In all cases where vit. E was added to the diets embryonic mortality during this period averaged about 8%. In the unsupplemented basal treatment and in the treatment with fish oil and DPPD alone, mortality during this period averaged about 13.5%. In previous reports by Jensen (1) and Atkinson *et al.*(2), the major difference between vit. E deficient and vit. E supplemented lots was in the embryonic mortality from the 25th to the 28th day of incubation. The fact that DPPD alone did not reduce the embryonic mortality during this period to a level equivalent to vit. E supplementation would suggest that the antioxidant was not completely effective in bringing about maximum hatchability. This may have been due to an inadequate level of the antioxidant. It is also possible that the antioxidant was only capable of protecting the vit. E naturally present in the basal diet. If this is the case, one would not expect the results with the antioxidant to be any better than those with the unsupplemented basal diet.

The analysis of egg yolks from the various treatments showed that alpha tocopheryl acetate added to the unsupplemented basal diet gave a marked increase in total tocopherol content of yolks of eggs laid by these hens. The addition of pollack liver oil to the basal diet caused a marked reduction in tocopherol content of the yolks. The addition of vit. E, DPPD, or a combination of the two with fish liver oil slightly increased the tocopherol content of the yolks, but did not bring it back to the level of the unsupplemented basal diet. This was surprising, since the hatchability of these eggs from hens supplemented with vit. E was as high as eggs from hens fed the basal diet plus vit. E without fish oil and was higher than eggs from hens fed the unsupplemented basal diet. The results suggest that vit. E and DPPD may be protecting some other unknown nutrient or metabolite which is essential for reproduction in the turkey. Bunnell *et al.*(6) proposed that the protective action of DPPD against encephalomalacia in chicks might be due to a stimulating effect on the metabolism of the unsaturated fats rather than a purely vit. E protective action.

Summary. Fish liver oil added to the diet greatly accentuated a deficiency of vit. E in turkey breeders. A reduction in fertility due to a vit. E deficiency was suggested. Both alpha tocopheryl acetate and DPPD counteracted the deleterious effect of fish oil on hatchability. With fish oil in the diet, tocopherol levels of the yolks of eggs were not appreciably increased by either vit. E or DPPD even though hatchability was near maximum.

1. Jensen, L. S., *Proc. Cornell Nutr. Conf.*, Cornell Univ., Ithaca, N. Y., 1953, 62.

2. Atkinson, R. L., Ferguson, T. M., Quisenberry, J. H., and Couch, J. R., *J. Nutrition*, 1955, v55, 387.

3. Ferguson, T. M., Atkinson, R. L., and Couch, J. R., PROC. SOC. EXP. BIOL. AND MED., 1954, v86, 868.

4. Singsen, E. P., Bunnell, R. H., Matterson, L. D., Kozeff, Anna, and Jungherr, E. L., *Poul. Sci.*, 1955, v34, 262.

5. Singsen, E. P., Matterson, L. D., Koseff, Anna, Bunnell, R. H., and Jungherr, E. L., *ibid.*, 1954, v33, 192.

6. Bunnell, R. H., Matterson, L. D., Singsen, E. P., and Eaton, H. D., *ibid.*, 1955, v34, 1184.

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Cultivation of Human Epithelial Cells in Tissue Culture.* (22271)

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(With technical assistance of S. Robert Wilson.)

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Several investigators have studied serial propagation of normal cells in tissue culture, particularly in the last 3 years, partly because of need for a substitute for fresh monkey kidney cells in mass production of poliomyelitis vaccine. Many other uses can be envisaged. Chang(1,2) accomplished this and 2 cell lines established by him from presumably normal human tissues were received in this laboratory early in 1955. This communication describes our experience in serial subcultivation of those 2 cell lines to determine their adaptability to various laboratory technics or use in diagnostic and investigative studies. Data are presented on media requirements, growth in various culture vessels, preservation at reduced temperatures, lyophilization, adaptation to growth in nonhuman sera, and treatment of contaminated cultures. The use of these cells for propagation of various viruses will be presented later.

Materials. Cell lines. Human conjunctiva had been originally removed from the bulbar conjunctiva of a child at strabismus operation, Feb. 17, 1954. The tissue was then considered nonmalignant. When received in this laboratory Feb. 3, 1955 it was in the 37th serial tissue culture passage. The human kidney had been removed from a hydrocephalic child at the time of subarachnoid ureteral anastomosis on 5-12-54. The kidney was then considered normal on gross examination. When received in this laboratory on 2-3-54, the cell line was in the 25th serial tissue culture passage. *Media.* Human, horse, calf, monkey, rabbit, and chicken sera were used in these experiments. Solution 199 (3) and Hank's balanced salt solution were prepared in the usual way. Eagle's medium (EM)(4) was prepared in 1x concentration and neutralized with 4.4% sodium bicarbonate prior to use. Soybean trypsin inhibitor was prepared in a 1% stock solution in Hank's balanced salt solution. Trypsin (Difco 1:250) was prepared in a 0.125% solution in phosphate buffer solution (pH 7.6). The above solutions were sterilized by sintered glass filtration. Chick embryo extract was prepared fresh weekly from 9 day viable embryos. The standard medium for cultivation of cells was Chang's basic medium (CM), *i.e.*, 20% human serum, 5% chick embryo extract, 75% balanced salt solution, and 0.001% crystalline soybean trypsin inhibitor. Antibiotics were added to a final concentration of 100 units per ml of penicillin, 100 μg of streptomycin, and 25 μg of nystatin.

Results. The 2 cell strains received were cultivated in 3 basic media to which were added varying percentages of human or animal sera. No difficulty was encountered in growing these cells. To evaluate quantitatively ability of these media and sera to support proliferation of the 2 cell strains, the cell count method described by investigators (2,5-8) was modified to fit our needs, *viz.*, preparation of standard cell suspension for inoculation by trypsinizing the cells growing in a milk dilution bottle culture. Total viable cells were enumerated by inclusion of 1:50000 neutral red in the trypsin, dispersing cells by gentle agitation and counting in a hemocytometer. Trypsin was removed after centrifugation at 1000 rpm for 10 minutes in International No. 1 centrifuge. The sedimented cells were resuspended in balanced salt solution and homogenously dispersed by a magnetic stirrer. Replicate roller tubes were inoculated with equal number of cells contained in 0.25 ml of suspension using automatic syringe and 4 tube cultures were fed with each medium. Replicate cultures were incubated at 37°C and refed with the same medium after 4 days. After 7 days incubation cells from replicate tubes were removed by trypsin containing 1:50000 neutral red,

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TABLE I. Effect of Animal Sera on Proliferation of Human Conjunctiva Cells Cultivated in Chang's Medium plus 20% Serum.

Serum	Avg No. cells per tube × 10^3	Proliferation index (7 days)
Horse 1	0*	0
2	0†	0
3	49	7.4
4	41	6.2
Calf 1	47	7.1
Rabbit 1	6.7	1.0
2	17.2	2.6
Sheep 1	14	2.1
Chick	21	3.1
Control (human #20)	77	11.6

* Inoculum/tube = 6.6 × 10^3 cells of Passage C_{55}.
† 0 indicates that all original cells inoculated into tubes were degenerated or lysed.

pooled, dispersed, and enumerated. Two separate cell counts were done for each pool of 4 cultures. A standard human serum was included in each test for comparison. Human sera from 38 separate donors were employed. Because of Chang's early experience (1) that 1 out of 5 human sera were toxic for conjunctiva cells and 2 out of 5 supported multiplication slowly, great pains were taken by us to collect these various sera carefully and in large quantity so that if a good serum was obtained enough would be on hand to carry the cells for a number of passages.

Only 2 of the 38 sera proved to be toxic for the tissue culture cells; both were toxic to both conjunctiva and kidney cells. Toxicity consisted of rounding and clumping of cells. All other sera were non-toxic and maintained both cell strains in normal morphologic state of clear epithelial-like sheets.

Sera from different donors varied greatly in ability to support cellular multiplication. The proliferation index (number of cells at end of 7 days ÷ number of cells inoculated) varied from 5.4 to 9 for conjunctiva and from 2.3 to 18.2 for kidney cells when different human sera were employed in Chang's medium. Sera from various animals varied in ability to support multiplication of conjunctiva cells when substituted for human sera in CM (Table I). Horse and calf sera seemed superior to rabbit, sheep, and chick sera tested but were not as good as human serum control. Two of the 4 horse sera tested were toxic.

Multiplication of conjunctiva and kidney cells when cultivated in CM, EM, and 199 media with added human, horse, or calf serum in 10 and 20% concentrations is shown in Tables II and III. These data indicate that both cell types multiplied faster in media with 20% serum than with 10% serum. Conjunctiva cells multiplied fastest in solution 199, CM and EM in that order. EM with human serum supported good growth but with horse or calf serum it was clearly inferior. Kidney cells responded in a similar manner. Beginning June 1955 efforts were made to adapt both cell lines to media containing horse and calf serum and no human serum. The adaptation was easily accomplished and conjunctiva have been carried since 6-24-55 for 18 serial passages from C_{50} to C_{68} in both 20% horse or calf serum in EM and 199 solution. Kidney cells have been serially subcultivated

TABLE II. Effect of Serum Concentration on Proliferation of Human Conjunctiva Cells Cultivated in Chang's, Eagle's, or 199 Medium with Added Human, Horse or Calf Serum.

Media	Avg No. cells per tube × 10^3		Proliferation index (7 days)	
	10% serum	20% serum	10%	20%
Chang's + human serum (21)	42*	54	7.0	9.
Eagle's human (21)	31	55	5.1	9.
" horse	10	26	1.6	4.3
" calf	12	11	2.0	1.8
199 horse	16	66	2.6	11.
199 calf	27	66	4.5	11.

* Inoculum/tube = 6 × 10^3 cells of Passage C_{56}.

TABLE III. Effect of Serum Concentration on Proliferation of Human Kidney Cells Cultivated in Chang's, Eagle's, or 199 Medium with Added Human, Horse or Calf Serum.

Media	Avg No. cells per tube × 10^3		Proliferation index (7 days)	
	10% serum	20% serum	10%	20%
Chang's + human serum (23)	13*	60	2.6	12
Eagle's human (23)	7	16	1.4	3.2
" horse	1.4	25	.28	5.0
" calf	1.1	4.4	.22	.88
199 horse	20	42	4.0	8.4
199 calf	24	64	4.8	12.8

* Inoculum/tube = 5 × 10^3 cells of Passage K_{48}.

TABLE IV. Total Cell Yield in Various Culture Vessels Inoculated with Either Human Conjunctiva or Kidney Cells.

Vessel (vol, ml)	Vol of media (ml)	Range of cell population after 7 days cultivation (human or horse serum) (× 1000)
Roller tube (18)	1	26 to 182
Milk dilution bottle (230)	10	250 to 3150
Blake bottle (1000)	50	1600 to 5800
Povitsky bottle (5000)	250-500	10000 to 30733

in these 4 media since 7-15-55 for 14 serial passages from K_{41} to K_{55}.

The 2 cell lines have been cultivated in microscopic glass chambers and hanging drop preparation for direct microscopy; roller tubes, milk dilution bottles, Blake bottles, and Povitsky bottles. In each type of culture vessel the range of cell population after approximately 7 days cultivation is shown in Table IV. Both types of tissue may be maintained in good morphologic condition for 2-3 weeks at 37°C by refeeding cultures twice weekly. For maintenance of conjunctiva cell cultures at a reduced metabolic rate they have been stored at 32°C, at 5°C, and at −70°C. Kidney cell cultures have been maintained at 32°C and 5°C. At 32° C cultures were stored with the usual volume of nutrient media and refed once weekly. At this temperature cultures maintained their usual morphologic state for 4-5 weeks. At 5°C they were stored with only a thin layer of nutrient media for 2 months. Conjunctiva roller tube cultures were quick frozen in acetone-CO_2 bath after removal of supernatant fluid, stored 2 days and revived upon feeding and incubation at 37°C. Eleven such conjunctiva tubes were lyophilized and stored at room temperature for one day. When they were refed and incubated at 37°C for 5 weeks with medium change once a week, 3 of the 11 cultures revived and formed sheets of new cells.

Centrifugally sedimented conjunctiva cells without added fluid were alternately quick frozen and thawed 10 times. When tested for viability, 1 of 4 cultures grew a sheet of cells in 7 days. When kidney cells were suspended in EM plus 20% horse serum in concentration of 1000000 cells/ml and then frozen and thawed 10 times, no viable cells were noted upon subsequent cultivation.

Non-viable cell extracts of both conjunctiva and kidney cells were prepared by this latter method. These were each injected into monkey kidney tissue cultures, 10 embryonated eggs, 4 rabbits, 4 guinea pigs and 10 mice to note presence of herpes virus simiae, lymphocytic choriomeningitis virus, tubercle bacilli, bacteria or any orphan virus which would manifest itself in these tests. No agents were found in either cell line by any of these tests.

Contamination of a batch of monkey tissue cultures is disappointing but economically not too serious because they can be discarded and duplicated within a week by setting up new tubes with fresh monkey kidney. However, bacterial or mold contamination is a severe threat to an established strain of cells which may have taken months to establish in serial tissue culture. To overcome this, we have applied the principles used in clinical medicine. The tissue culture fluid is streaked on agar plates and antibiotic discs added. The antibiotic of choice is added to tissue culture medium for several passages. Cultures contaminated with molds or yeasts have been successfully treated by adding 50 μg of nystatin for several passages.

To note the virus yield from poliomyelitis stool specimens inoculated into tissue cultures of conjunctiva cells as compared to tissue cultures of monkey kidney cells, 2 stool specimens from poliomyelitis patients were inoculated into 3 roller tube cultures each of conjunctiva and monkey kidney. Cytopathogenicity occurred after 1 day in monkey kidney cultures and after 2 days in conjunctiva cultures. After 5 days, supernates from each cell type were pooled and typed with specific antisera and titrated in monkey kidney cells to determine the virus titer in the fluids. The poliovirus isolated was Type I in stool A and the virus titer in the 5-day supernate from monkey kidney cells was $10^{6.33}$ per ml as compared to $10^{6.41}$ per ml from conjunc-

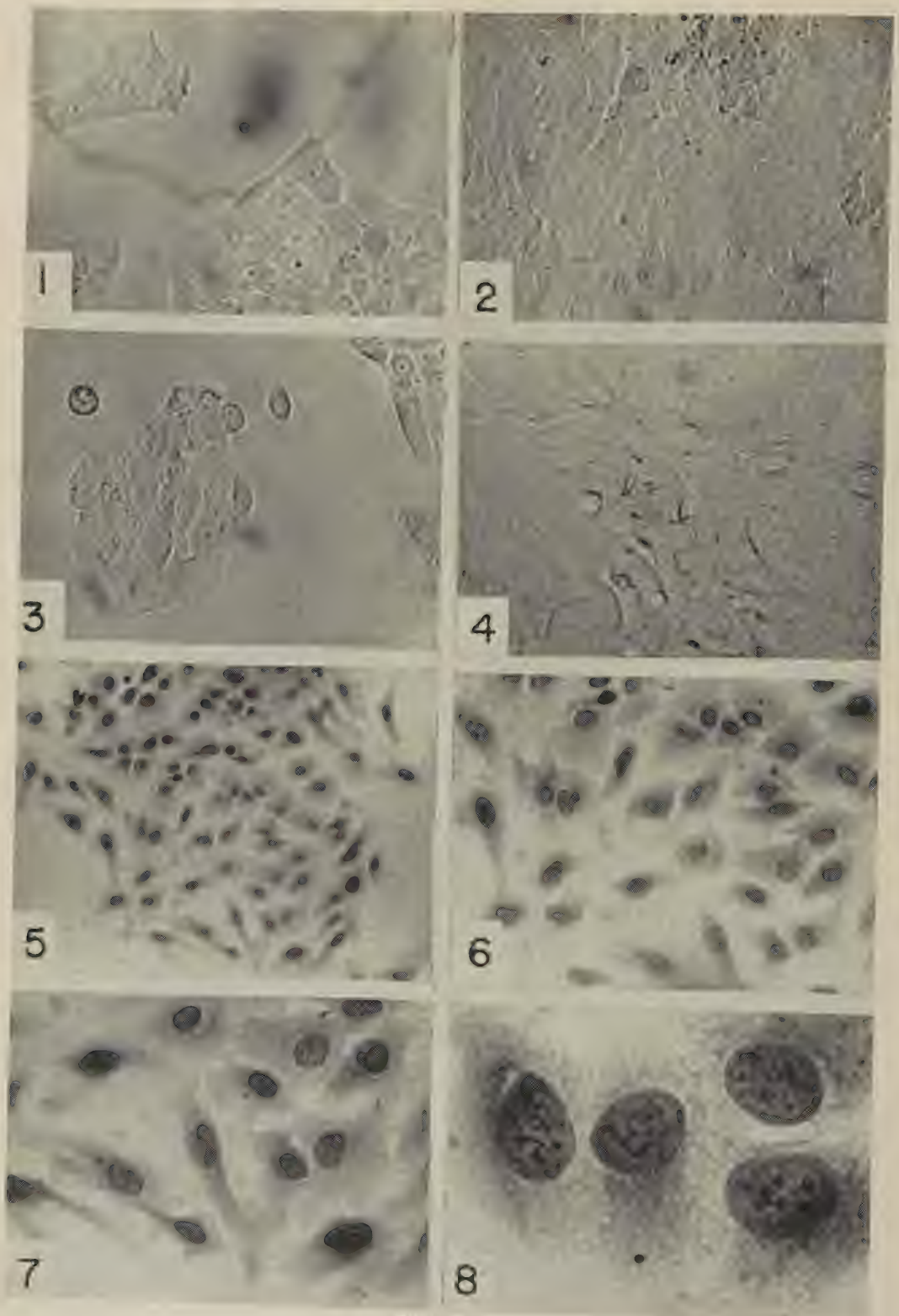
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FIG. 1. Conjunctiva cells, 2 days after subcultivation showing islands of flat monolayer epithelial cells. Unstained. × 220.

FIG. 2. Conjunctiva cells, 10 days after subcultivation showing thick sheet of cells with some clumping. Unstained. × 190.

FIG. 3. Kidney cells, 2 days after subcultivation showing islands of cells. Unstained. × 220.

FIG. 4. Kidney cells, 7 days after subcultivation showing thick sheet of cells. Individual cell borders are indistinct because of light diffraction by underlying cells. Unstained. × 190.

FIG. 5-8. Conjunctiva cells, 2 days after subcultivation, showing cellular and nuclear detail. Magnification × 100, 250, 400, and 1000, respectively. Osmic acid fixation, toluidine blue stain.

tiva cells. The virus isolated was Type II in stool B and the virus titer in the 5th day supernate from monkey kidney cells was $10^{6.71}$ per ml as compared to $10^{6.76}$ per ml from conjunctiva cells.

Histology. The morphologic appearance of these 2 cell lines is shown in Plate 1. Both produce large epithelial-like cells with a nuclear cytoplasmic ratio of about 1-6. The cells fit together in flat monolayer sheets in young cultures but form thick sheets, sworls, and thick clumps in older cultures. There is remarkable uniformity of cells although occasional abnormal mitotic figures are observed and rarely a binucleate cell. Polyploidy has been observed but its significance cannot be interpreted in the present state of knowledge of how cells behave in tissue culture. It is of interest that the microscopic appearance of these cells has not changed since the original publication by Chang(1).

Discussion. It is believed that these cell lines established by Chang represent the first human cells derived from normal tissues and successfully cultivated serially *in vitro* over a prolonged period of time. As such, they offer a research tool of great potential usefulness in study of virus diseases and other problems.

These cells can be grown in a variety of containers for microscopic studies or for mass production or chemical analysis. They thrive in media free from human serum and in relatively simple synthetic media. The toxicity of many human sera for these cells, observed by Chang during earlier subcultures, has not been observed by us, nor has it been observed by Chang in recent months. Presumably this is a reflection of a change in the cells, the nature of which is still quite obscure. The cells can be stored for prolonged periods at low temperature and some will survive freezing and lyophilization. Tests for presence of a resident virus in these cultures have been negative, and sublines have been maintained for many generations without any human serum added. This was done to prevent contaminating cultures with a hepatitis virus or other human pathogenic agent(9).

In very limited studies one of the cell lines appeared to be as sensitive as monkey kidney cells for the isolation of poliomyelitis viruses. If this proves to be true, they might be very useful for laboratory diagnosis. This is now being tested. Because of the ease of handling these cells and because they are of human origin, they should be useful in the search for a laboratory method to grow viruses which as yet have not been cultivated in a practical manner such as measles and German measles. Other diseases such as infectious mononucleosis, infectious polyneuritis, mesenteric adenitis and hepatitis may be explored with such cell lines.

Since the conjunctiva and kidney cells grow so well in tissue culture, one might ask whether they have also acquired the power of independent growth *in vivo*. Studies are under way using the technics of Greene(10) of implantation in the brain and anterior chamber of guinea pigs, and the technics of Toolan(11-14) and Moore(15) of inoculation of irradiated rats.

Summary. 1. Experience is described in the serial subcultivation of 2 human cell lines derived from conjunctiva and kidney and established in serial passage *in vitro* by Chang. 2. Thirty-eight human sera were compared in their ability to support multiplication of the 2 cell lines. Two of the sera were toxic to both cell lines. The remaining 36 sera were non-toxic but varied in their ability to support cellular multiplication. Horse and calf

sera were superior to rabbit, sheep, and chicken sera but inferior to the human serum control. 3. Comparison was made of the multiplication of the 2 cell lines when cultivated in Chang's, Eagle's, and 199 media with added human, horse, or calf serum in 10 and 20% concentration. 4. Conjunctiva cells have been serially subcultivated for 18 passages in media containing horse or calf serum but no human serum. Kidney cells have been carried for 14 passages in such media. 5. Conjunctiva and kidney cells survived for varying periods when stored at 32°C or 5°C. Some sedimented conjunctiva cells survived 10 freezing-thawing cycles and formed a sheet of cells when incubated at 37°C. 6. Three of 11 conjunctiva cultures survived lyophilization and revived when reincubated at 37°C for 5 weeks. No contaminating bacterium or virus could be detected in these cell lines by the injection of nonviable cell extracts into tissue cultures of monkey kidney, embryonated eggs, rabbits, guinea pigs, and mice. 7. A method is described for the treatment of contaminated tissue cultures. 8. The conjunctiva cells gave results comparable to monkey kidney cells when used for isolation of poliomyelitis virus from 2 stool specimens.

1. Chang, R. S., PROC. SOC. EXP. BIOL. AND MED., 1954, v87, 440.
2. ———, *Trans. N. Y. Acad. Sc.,* 1955, v17, 584.
3. Morgan, J. F., Morton, H. J., and Parker, R. C., PROC. SOC. EXP. BIOL. AND MED., 1950, v73, 1.
4. Eagle, H., *J. Exp. Med.,* 1955, v102, 37.
5. Sanford, K. K., Earle, W. R., Evans, V. J., Waltz, H. K., and Shannon, J. E., Jr., *J. Nat. Cancer Inst.,* 1951, v11, 773.
6. Scherer, W. F., Syverton, J. T., Gey, G. O, *J. Exp. Med.,* 1953, v97, 695.
7. Owens, O. H., Gey, M. K., and Gey, G. O, *Ann. N. Y. Acad. Sc.,* 1954, v58, 1039.
8. Youngner, J. S., PROC. SOC. EXP. BIOL. AND MED., 1954, v85, 202.
9. Buetler, E., and Dern, R. J., *J.A.M.A.,* 1955, v159, 989.
10. Greene, H. S. N., *Science,* 1938, v88, 357.
11. Toolan, H. W., *Cancer Research,* 1954, v14, 660.
12. ———, PROC. SOC. EXP. BIOL. AND MED., 1954, v86, 607.
13. ———, *Ann. N. Y. Acad. Sc.,* 1955, v59, 394.
14. ———, *Trans. N. Y. Acad. Sc.,* 1955, v17, 589.
15. Moore, A., personal communication.

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Serial Biochemistry of Serum and Cerebrospinal Fluid in Amaurotic Family Idiocy (Tay-Sachs Disease).* (22272)

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Studies in Amaurotic Family Idiocy (AFI) have thus far been principally concerned with histologic examination and biochemical analysis of brain tissue. These latter investigations resulted in isolation of a new cerebroside-like substance called a "ganglioside" and differing from other cerebrosides by the presence of neuraminic acid(1-3). In contrast to other lipoidoses, it is generally assumed that there are no characteristic serum changes in AFI.

The present report is concerned with serial studies of the neuraminic acid, protein and lipid fractions in serum, and cerebrospinal fluid (CSF) proteins in a group of 7 children with infantile AFI, the diagnoses in most instances confirmed at autopsy. Similar biochemical studies of 2 children with Schilder's disease and gargoylism, serving as controls, were also carried out. Most children in this survey were admitted to this institution shortly after clinical inception of the disease and remained hospitalized until after death. The increased longevity of some patients with AFI in this study(4), afforded greater opportunity for carrying out such serial biochemical

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TABLE I. Serum and C.S.F. Protein Data in Amaurotic Family Idiocy and Related Disorders.

Diagnosis	T.P., g %	Alb., g %	Alpha-1, g %	Alpha-2, g %	Beta, g %	Gamma, g %	A/G, %
	Serum electrophoretic data						
Tay-Sachs disease, 7 p, 20 d*	6.80 ± .35† <.01‡	3.70 ± .33 <.01	.50 ± .09 .40	.98 ± .17 <.01	.96 ± .13 —	.65 ± .21 <.01	1.20 ± 2.10 .15
Schilder's disease, 1 p, 3 d	7.39§	3.50	.59	.95	1.28	1.08	.90
Gargoylism, 1 p, 4 d	7.12§	3.57	.52	.95	1.25	.83	1.00
Normal subjects	7.26 ± .39	4.07 ± .29	.48 ± .09	.66 ± .11	.96 ± .20	1.01 ± .20	1.30 ± .18

Diagnosis	T.P., mg %	Alpha, mg %	Alpha/T.P., %	Gamma, mg %	G.G./T.P., %	Serum neuraminic acid, mg %
	C.S.F. proteins (chemical)					
Tay-Sachs disease, 7 p, 20 d*	41.7 ± 11.32† .10‡	5.9 ± .94 <.01	16.2 ± 3.11 .045	1.7 ± .98 <.01	4.9 ± 2.88 <.01	89.6 ± 7.10 <.01
Schilder's disease, 1 p, 3 d	51.6§	7.9	15.2	9.5	21.3	82.7
Gargoylism, 1 p, 4 d	50.8§	9.5	18.7	3.4	6.6	84.4
Normal subjects	37.5 ± 5.0	8.6 ± 1.2	18.2 ± 2.2	4.0 ± 1.0	9.4 ± 1.8	72.9 ± 7.4

* p = patients; d = determinations. † Mean ± S.D. ‡ P. § Mean.

analyses.

Methods. (a) *Serum:* The procedure used for the electrophoretic protein determinations has been described(5,6). Total proteins were determined by biuret procedure(7) with frequent checks against the micro-Kjeldahl method. Total lipids were determined gravimetrically with the Bloor(8) method; phospholipids with the modified colorimetric procedure of Youngburg and Youngburg(9) and free fatty acids by the titrimetric technic of Kaiser and Kagan(10). Total and free cholesterol values were obtained with colorimetric method of Zak, *et al.*(11) and cholesterol esters and cholesterol ester/total cholesterol ratios were calculated from these experimental results. Neutral fats were calculated from the above data by the formula given in the article by Goldbloom(12). Lipase values were determined by the method of Goldstein, *et al.*(13) and neuraminic acid by a modification of the procedure described by Winzler (14). (b) *Cerebrospinal Fluid*: Total proteins were determined by a modification of the Weichselbaum biuret method(15). Gamma globulins were determined by quantitative protein flocculation-ninhydrin colorimetric procedure(16) and alpha globulins with a newly developed turbidimetric method(17) as modified from a previously described technic of Jacox(18) for serum alpha globulins. The simultaneous serial studies on both serum and CSF were generally carried out in duplicate at bi-monthly intervals for periods to 10 months. While the derived biochemical data from the AFI cases were *statistically* analysed (Tables I, II) it was deemed unnecessary to apply such an evaluation to the two control cases because of insufficient number of determinations.

Results. Previous experience with other neurological entities of a degenerative nature such as multiple sclerosis, Schilder's disease, amyotrophic lateral sclerosis, etc., has shown consistent alterations of both the serum and CSF protein fractions as determined by electrophoretic and biochemical technics. The significance of these changes is not related to alterations in the individual protein fractions, but rather in the entire "protein profile"(19,20). For example, while there is a notable increase in CSF gamma globulin in multiple sclerosis, no corresponding rise in serum gamma globulin is evident, in contrast to many infectious types of disease, *e.g.*, neurosyphilis, wherein both CSF and serum gamma globulin fractions rise above the anticipated normal.

TABLE II. Serum Lipid Data in Amaurotic Family Idiocy and Related Disorders.

Diagnosis	Serum total lipids	Serum neutral lipids	Serum phospholipids	Serum free fatty acids	Total cholesterol	Free cholesterol	Cholesterol esters	Ratio, %	Serum lipase, units
	mg %								
Tay-Sachs disease, 4 p, 15 d*	675.0 ± 137.1† <.01‡	221.0 ± 58.4 <.01	155.5 ± 37.0 <.01	332.0 ± 56.6 .48	192.5 ± 41.75 .018	41.8 ± 12.04 <.01	151.0 ± 32.18 .22	78.4 ± 3.62 <.01	1.08 ± .512 .28
Gargoylism, 1 p, 5 d	702.0§	175.0	182.0	382.0	271.0	55.0	215.0	79.4	1.15
Schilder's disease, 1 p, 3 d	954.0§	302.0	226.0	411.0	287.0	79.0	208.0	72.6	1.18
Normal subjects	861.0 ± 75.0	365.0 ± 59.0	218.0 ± 15.0	320.0 ± 35.0	220.0 ± 20.0	53.0 ± 7.5	162.0 ± 14.0	75.0 ± 2.5	1.23 ± .06

* p = patients; d = determinations. † Mean ± S.D. ‡ P. § Means.

In our series of 7 cases with AFI, a statistical analysis of chemical serum total protein and electrophoretically determined serum albumin showed significant decreases below normal levels. (Table I). In the 2 control cases, with remotely related degenerative diseases of CNS (Schilder's disease and gargoylism), a relatively normal total protein was noted, although serum albumin values were somewhat decreased. Alpha-2 serum globulin fractions were increased in AFI, in contrast to a marked decrease in serum gamma globulin component. Alpha-1 and beta globulin fractions did not vary significantly from normal values. A rather surprising finding was a normal A/G ratio in AFI, since it is frequently depressed in many degenerative diseases, irrespective of etiology. This is exemplified by lowering of this ratio in the case of Schilder's disease (Table I).

The biochemical studies on the CSF samples done simultaneously with the sera, and employing chemical methods, showed a relatively normal total protein but a lowered alpha globulin fraction and markedly decreased gamma globulin values, as well as diminished gamma globulin/total protein ratios. This decrease in CSF gamma globulin levels has not been observed in a wide variety of other progressive, and generally fatal, neurologic diseases and hence cannot be equated with debilitation alone. In most other CNS diseases (including Schilder's disease, gargoylism, multiple sclerosis, cerebral palsy, hemiplegia, etc.) this CSF protein component is either normal or elevated(20,21).

Because of the intimate association of neuraminic acid with AFI, studies of serum level of this complex amino acid were inaugurated. This substance has been identified as one of the abnormal components of the "ganglioside" which constitutes the anomalous dystrophic accumulation within the involved neurons in AFI(1-3). A definite elevation of neuraminic acid in our 4 cases of AFI was observed. This finding is in keeping with the elevation of this substance previously demonstrated in cerebral parenchyma in this disease(1,22,23). Since the 2 control cases also show an elevation in this component, the

specificity of this change in AFI as compared to other neurologic disorders is presently under investigation.

Previous biochemical studies on lipoid dystrophies have dealt mainly with variations in the lipid fractions of visceral organs(24). In a few of these diseases such lipid abnormality is also reflected in alterations of such fractions within the serum. No comparable changes of serum lipids have been reported in AFI to our knowledge. For this reason, biochemical studies of serum lipid partition within total lipids of this disease were performed (Table II). When such results were statistically compared to those derived from normal subjects of comparable age, some marked variations were noted. The total serum lipids, neutral lipids and phospholipids all showed a significant decrease. In the one case of gargoylism, directional changes of a similar nature were recorded. In the instance of Schilder's disease, however, normal or elevated values of these lipid fractions were noted. In studies of the cholesterol fractions, a slight decrease of free cholesterol and slight elevation in the cholesterol ester/total cholesterol ratio were seen. No significant changes were apparent in total cholesterol or cholesterol esters however. Serum lipase and free fatty acid levels were normal. Further studies concerned with lipid and protein metabolism in AFI are now in progress.

Summary. Serial biochemical serum and cerebrospinal fluid (CSF) studies in 7 cases of Amaurotic Family Idiocy (AFI) disclosed some *statistically* significant variation from normal values. Two additional control cases of dissimilar degenerative neurologic illnesses of childhood were also included as control cases. In AFI an elevation in serum electrophoretic alpha-2 globulin fraction was found. Decreased values for serum total protein and albumin, together with markedly depressed gamma globulin results were observed. Serum alpha-1 and beta globulins and A/G ratio findings were normal. Chemical studies of CSF proteins showed decreased alpha and gamma globulins and gamma globulin/total protein ratio values. Serum neuraminic acid was consistently elevated in AFI, as well as in the 2 control cases, when compared to normal values established in this laboratory. Decreases in serum total, neutral and phospholipids were recorded in AFI. A decrease was also observed in serum free cholesterol values. The results in AFI differed from the 2 control cases of degenerative central nervous system disease most markedly with respect to lowered alpha and gamma globulin content of the CSF and with regard to decreased serum total lipids and phospholipids.

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1. Klenk, E., *Z. physiol. Chem.,* 1941, v268, 50.
2. ———, *ibid.,* 1942, v273, 76.
3. ———, *ibid.,* 1951, v288, 216.
4. Aronson, S. M., Volk, B. W., and Epstein, N., *Am. J. Path.,* 1955, v31, 609.
5. Saifer, A., Rabiner, A. M., Oreskes, I., and Volk, B. W., *Am. J. M. Sc.,* 1953, v225, 287.
6. Volk, B. W., Saifer, A., Johnson, L. E., and Oreskes, I., *Am. Rev. Tuberc.,* 1953, v67, 299.
7. Gornall, A. G., Bardawill, C. J., and David, M. M., *J. Biol. Chem.,* 1949, v177, 751.
8. Bloor, W. R., *ibid.,* 1914, v17, 377.
9. Hawk, P. B., Oser, B. L., and Summerson, W. H., *Practical Physiological Chemistry,* 13th edition, Blakiston Co., New York, 1954, 589.
10. Kaiser, E., and Kagan, B. M., *Anal. Chem.,* 1951, v23, 1879.
11. Zak, B., Dickenman, R. C., White, E. G., Burnett, H., and Cherney, P. J., *Am. J. Clin. Path.,* 1954, v24, 1307.
12. Goldbloom, A. A., *Gastroenterology,* 1952, v20, 79.
13. Goldstein, N. P., Epstein, J., and Roe, J. H., *J. Lab. and Clin. Med.,* 1948, v33, 1047.
14. Winzler, R. J., *Methods of Biochemical Analysis,* Vol. II, D. Glick, ed., Interscience Publishers, New York, 1955, 296.
15. Weichselbaum, T. E., *Am. J. Clin. Path.,* Tech Sect., 1946, v10, 40.
16. Saifer, A., and Narby, T., *J. Lab. and Clin. Med.,* 1953, v42, 316.
17. Saifer, A., and Zymaris, M. C., *Clinical Chem.,* in press.
18. Jacox, R. F., *J. Clin. Invest.,* 1953, v32, 661.
19. Volk, B. W., Saifer, A., Rabiner, A. M., and Oreskes, I., *A. M. A. Arch. Neurol. and Psychiat,* 1955, v73, 66.
20. Volk, B. W., Saifer, A., Rabiner, A. M., and

Hinterbuchner, L. P., *ibid.*, in press.

21. Yahr, M. D., Goldensohn, S. S., and Kabat, E. A., *Ann. New York Acad. Sc.*, 1954, v58, 613.

22. Sperry, W. M., *J. Mt. Sinai Hosp.*, 1942, v9, 799.

23. Sobotka, H., *ibid.*, 1942, v9, 795.

24. Thannhauser, S. J., *Lipidoses*, Oxford Univ. Press, New York, 1950, 579.

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Chemotherapy of Leukemia VII. Effect of Substituted Triazenes on Transplanted Mouse Leukemia.* (22273)

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During routine screening of compounds for possible antitumor effects, a member of a new series of compounds not previously known to possess chemotherapeutic activity, 3,3-dimethyl 1-phenyl triazene (Triazene I), was found by Clarke *et al.*(1) to inhibit growth of Sarcoma 180 in mice. Following this Dagg, Karnofsky *et al.*(2) demonstrated teratogenic effects on the chick embryo and inhibition of growth of Sarcoma 180 and certain human tumors implanted on the chorioallantoic membrane. An evaluation of chemotherapeutic effects of this and related compounds against various strains of transplanted leukemia in mice has been undertaken and the results are herewith reported.

Method. The general technic used for evaluating chemotherapeutic activity of a given drug by means of its ability to prolong survival time of mice with transplanted leukemia has been described previously(3). The 60th to 70th transplant generations of leukemia 82(4), which originated as a spontaneous leukemia in a C58 mouse in October 1953, were used for most of these studies. This transplantable leukemia usually kills the mice in 10 to 15 days after inoculation, with an elevation of total leukocyte count to the 50000 to 200000 level, enlargement of liver and spleen, and some lymphadenopathy. A few studies were conducted on the 10th to 14th generation of leukemia 5471, which arose as a spontaneous leukemia in a C58 mouse in July, 1954. Although morphologically these 2 leukemias are very similar, 82 has always been relatively refractory to folic acid antagonists, whereas 5471 is quite sensitive. In these experiments mice of the F1 hybrid generation of C58 male X Bagg albino female cross were each injected intraperitoneally with 0.1 cc of saline suspension of minced spleen containing one million cells per inoculum. The A-methopterin sensitive (L1210S) and A-methopterin dependent (L1210AD) variants of Leukemia L1210 obtained from Law(5,6) were also used. These leukemias were transplanted by subcutaneous injections into dba mice of minced tumor tissue suspended in isotonic saline. Treatment was initiated 24 hours after inoculation and repeated 3 times weekly for 10 doses over a 23-day period. The mice were observed for development of leukemia and autopsied at death. Triazenes that have been adequately tested and their maximum tolerated doses are listed below. Because of low solubility in water, these compounds were either dissolved in peanut oil (PO) or suspended in 0.5% sodium carboxymethylcellulose (CMC) in isotonic saline as indicated:

I.	3,3-dimethyl-1-phenyl triazene	12-25 mg/kg (PO)
II.	3,3-diethyl-1-phenyl triazene	150 mg/kg (PO)
III.	3,3-dimethyl-1-p-nitrophenyl triazene	250 mg/kg (CMC)
IV.	3,3-dimethyl-1-p-tolyl triazene	40-62.5 mg/kg (CMC)

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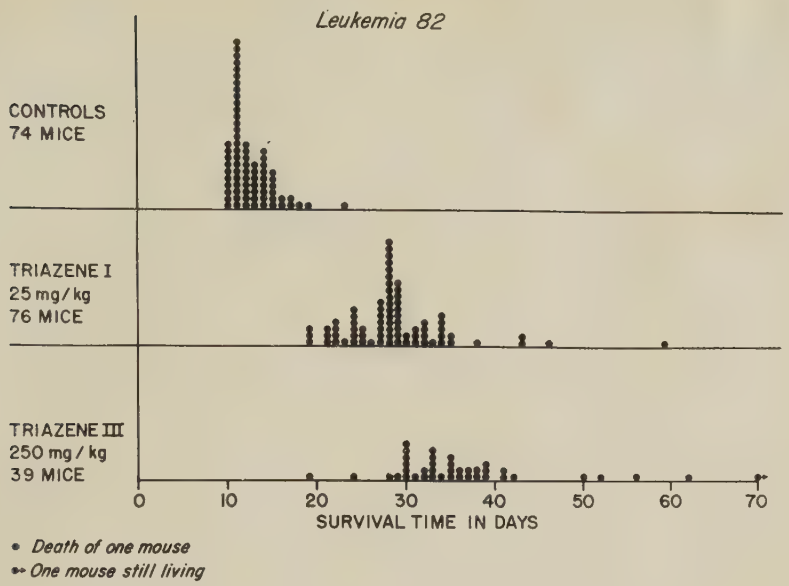

FIG. 1.

Results. The scatter diagram in Fig. 1 shows the relative effectiveness of triazenes I and III in leukemia 82. The mean survival time of the 74 control animals was 12.5 days whereas mice treated with Triazene I at a dose of 25 mg/kg of body weight showed mean survival time of 28.9 days and those receiving Triazene III at 250 mg/kg 35.8 days.

The comparative effectiveness of the various triazenes and various standard agents against leukemias 82, 5471, L1210S and L1210AD is demonstrated in Table I.

It can be seen that Triazene III was somewhat more active against leukemia 82 than Triazenes I and IV and that the diethyl triazene (II), was not as effective.

The triazenes were without therapeutic activity against L1210S and L1210AD. In other experiments using variants of leukemia 82 made resistant to amicetin(7), azaserine (8) or aminonucleoside(7), Triazene I was still effective in prolonging survival time.

Using a technique previously reported(9), massive single doses of Triazene I of 1000, 2000, 4000, and 8000 mg/kg of body weight (5, 10, 20 and 40 times the acute LD_{50} dose respectively) were injected intraperitoneally into mice with far advanced 82 leukemia. Bioassay of spleens from these animals into young mice of the same strain 1-2 hours after injection produced leukemia at all levels. This result is in contrast to the sterilizing effects in the same experiment of single injections of 2 to 10 times the LD_{50} dose of one of the more potent nitrogen mustards 1, 2, 3, 4, tetrakis (bis [2-chloroethyl] amino) butane tetrahydrochloride(9,10) and in contrast to the previously reported sterilizing effect of triethylene melamine (TEM) and other alkylating agents on various strains of mouse leukemia.

Discussion. It has been postulated that the chemotherapeutic activity of triazenes is due to their action as alkylating agents. In this respect it should be noted that they are active on strains of transplantable mouse leukemia (82 and 5471) which are also inhibited by the nitrogen mustard methyl-bis (β-chloroethyl) amine (HN2) but that although Triazene I prevents the transplantability of Sarcoma 180 growing on the chorioallantoic membrane of the chick embryo(2) no sterilizing effect could be demonstrated in mouse leukemia in contrast to the activity of HN2, TEM, and other alkylating agents in this respect. Thus it is evident that certain differences as well as similarities exist between the triazenes and the typical alkylating

TABLE I. Comparative Effects of Triazenes on Various Strains of Mouse Leukemia. Mean survival time in days and range.

	Dose, mg/kg	Leukemia 82				Leukemia 5471	Leukemia L1210S	Leukemia L1210AD
		Exp. 1	Exp. 2	Exp. 3	Exp. 4			
Controls	—	11.2 (11–12)	14.9 (12–17)	14.3 (12–19)	11.8 (10–14)	15.2 (14–17)	13.3 (12–14)	15.4 (8–20)
Triazene I	25	27.8 (22–38)	31.4† (33–50)	30.4 (24–43)	24.1 (19–29)	32.3 (24–49)	13.9 (12–15)	16.4 (13–20)
	12.5	34.2* (22–50)	37.2* (30–50)	42.6‡ (29–50)	39.7 (33–45)	—	—	15.7 (9–20)
II	150	15.5 (12–18)	—	—	—	—	—	—
III	250	34.4* (19–50)	—	—	43.2 (33–62)	—	14.2 (14–15)	14.8 (9–20)
IV	62.5	21.5 (15–28)	—	—	—	—	—	—
	40	—	35.6 (27–44)	—	—	—	—	—
HN2	1	19.0 (12–22)	—	24.6 (22–28)	—	—	—	13.9 (11–17)
Urethane	1000	—	—	22.3 (20–27)	27.3 (20–34)	22.7 (19–28)	—	—
A-Methopterin	3	—	—	—	—	45.7 (35–84)	26.1 (21–37)	13.5 (11–16)

* 1 living at 50 days. † 2 living at 50 days. ‡ 3 living at 50 days.

agents.

In comparative studies on triazene derivatives, addition of the nitro group in the para position of the phenyl group (III) decreased markedly the toxicity of the compound for the host mouse and increased somewhat its relative chemotherapeutic effectiveness against leukemias 82. It was not an effective agent however, against the Amethopterin sensitive or Amethopterin resistant variants of leukemia L1210 which were also refractory to the parent compound (I). Lack of effect of this derivative (III) on development of the chick embryo was attributed by Dagg *et al.* (11) to the fact that in the 4-day-old chick embryo in contrast to the mouse, the addition of the nitro group in the para position of the phenyl group increased rather than decreased the overall toxicity of the compound and thus perhaps made it impossible to give large enough doses to demonstrate a teratogenic effect.

The fact that these triazenes were active against Leukemia 82 which is initially refractory to treatment with Amethopterin and also against lines of Leukemia 82 made resistant to Azaserine, Amicetin, or Aminonucleoside indicates that these compounds are acting through a different mechanism in producing their chemotherapeutic effects.

The above mentioned studies of Clarke *et al.*(1) of Dagg, Karnofsky, *et al.*(2) and the presently reported studies on mouse leukemia suggest that this new series of compounds be given clinical evaluation against neoplastic disease. Such studies are now in progress.

Summary. 1-phenyl 3, 3-dimethyl triazene, has been demonstrated to prolong survival time of mice with transplanted Leukemias 82 and 5471 but not with an Amethopterin sensitive or an Amethopterin resistant variant of Leukemia L1210. A closely allied derivative, 1 - p - nitrophenyl - 3,3-dimethyl triazene has shown relatively greater chemotherapeutic activity against Leukemia 82 but was inactive against the two variants of Leukemia L1210. These compounds were also active against strains of Leukemia 82 which were either initially refractory to or made resistant to Amethopterin, Azaserine, Amicetin, and Aminonucleoside. Further therapeutic studies on this class of compounds are indicated.

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1. Clarke, D. A., Barclay, R. K., Stock, C. C., and Rondestvedt, C. S., Jr., PROC. SOC. EXP. BIOL. AND MED., 1955, v90, 484.

2. Dagg, C. P., Karnofsky, D. A., Stock, C. C., Lacon, C. R., and Roddy, J., *ibid.*, 1955, v90, 489.

3. Burchenal, J. H., Johnston, S. F., Burchenal, J. R., Kushida, M. N., Robinson, E., and Stock, C. C., *ibid.*, 1949, v71, 381.

4. Burchenal, J. H., Yuceoglu, M., Dagg, M. K., and Stock, C. C., *ibid.*, 1954, v86, 891.

5. Law, L. W., Dunn, T. B., Boyle, P. J., and Miller, J. H., *J. Nat. Cancer Inst.*, 1949, v10, 179.

6. Law, L. W., and Boyle, P. J., PROC. SOC. EXP. BIOL. AND MED., 1950, v74, 599.

7. Burchenal, J. H., Dagg, M. K., Wiegand, L. S., unpublished observations.

8. Murphy, M. L., Dagg, M. K., and Burchenal, J. H., to be published.

9. Burchenal, J. H., Cremer, M. A., Williams, B. S., and Armstrong, R. A., *Cancer Research*, 1951, v11, 700.

10. Burchenal, J. H., Burchenal, J. R., and Johnston, S. F., *Cancer*, 1951, v4, 353.

11. Dagg, C. P., and Karnofsky, D. A., personal communication.

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Effect of Adrenalin on Resistance of Adrenalectomized Rats to Certain Toxic Agents. (22274)

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Prior to 1917, the secretion of the adrenal medulla was believed to be the entity which rendered the adrenal glands indispensable for maintenance of life(1). Following the demonstration that epinephrine-free cortical extracts maintained normal blood sugar levels in adrenalectomized rats(2), the role of the medullary hormone has generally been regarded as of minor importance, if any. In searching for a rapid method of assay for DOC-like activity of steroids, it was observed that adrenalin rather than cortical steroids conferred resistance to administration of certain toxic agents in adrenalectomized rats. The sequence of events which led to this finding is here described. On the premise that adrenalectomized animals exhibit decreased tolerance to administered potassium(3), the proposed method of assay was to determine the amounts of cortical steroids which would provide increased resistance to the injection of potassium chloride.

Methods. Intact and adrenalectomized male albino Sprague-Dawley rats, one-half of each group having been treated with desoxycorticosterone acetate, 1 mg rat/day up to 7 days, were injected subcutaneously with 0.5 cc of 10% potassium chloride solution every 10 minutes until death, and the number of injections given up to the time of death was recorded. It was noted that adrenalectomized animals succumbed after only one-half as many injections as those given intact animals, and that even intensive pretreatment with DCA did not diminish the susceptibility of adrenalectomized rats to injected potassium chloride.

Results. Since, in some of the adrenalectomized rats, convulsions suggestive of hypoglycemic shock were noted after only one or 2 injections of KCl, glucose determinations were made on blood collected from the heart at the instant of respiratory failure. These values are shown in Table I.

TABLE I. Blood Sugar of Intact and Adrenalectomized Rats Injected with KCl.

Blood sugar (mg %)*	Untreated	KCl-treated
Intact	135	275
Adrenalectomized	85	45

* Pool of 4 animals.

The blood sugar values confirmed the impression that adrenalectomized rats were indeed in a hypoglycemic state, and also showed that intact animals exhibited an increase in

TABLE II. Effects of Adrenalectomy, Adrenal Demedullation, and Administration of DCA, Cortisone, and Adrenalin on Sensitivity to KCl.

No. of inj.	4	5	6	7	8	9	10	11	12	13	14	15	16	Blood sugar (mg %) Initial	Terminal
No. of deaths:															
Intact			2	3	4		1	2						130	270
Adx.	5	3	1	2	1									115	40
" + DCA	4	3		3	2									155	80
" + cortisone	1	1	3	3	2	1	1							135	75
Demedullated	1	2	2	3	3			1						—	125
Adx. + adrenalin				2	4	2			1	2			1	110	240

blood sugar. It was thereupon decided to investigate whether the hypoglycemic response of the adrenalectomized rats could be modified by the administration of glucocorticoids, and if so, whether their sensitivity to KCl could thereby be reduced. In addition, since the ablation of adrenals deprives the animal of another hyperglycemic factor, the medullary secretion, the study was extended to include the effect of adrenalin administration. One group of adrenalectomized rats was treated for 7 days with 1 mg cortisone acetate injected subcutaneously/rat/day. On the 8th day they were injected with KCl as in the foregoing test. Another group of adrenalectomized rats was injected with a solution of KCl, each 0.5 cc containing 10 γ of adrenalin chloride. A third group consisted of rats whose adrenals had been enucleated 1 month previous to the test. The remaining groups included DCA-treated adrenalectomized rats, and untreated intact and adrenalectomized controls. Blood sugars were collected terminally.

The results (Table II) confirmed the previous findings that untreated adrenalectomized rats tolerated less KCl than intact animals and that DCA-treated adrenalectomized rats showed the same sensitivity to KCl as untreated adrenalectomized animals. They also demonstrated that cortisone-treated adrenalectomized rats and demedullated rats were only slightly less sensitive to KCl than adrenalectomized animals and that adrenalin-treated adrenalectomized rats tolerated KCl as well as or possibly better than did intact rats. Hyperglycemia was present in the intact and in the adrenalin-treated rats; the cortisone- and DCA-treated groups and the demedullated rats showed a lower blood sugar level than the intact rats. The untreated adrenalectomized animals exhibited hypoglycemia.

In view of the protection given by adrenalin, this treatment was tested against 2 other arbitrarily selected toxic agents, formaldehyde and sodium arsenite. The former was injected subcutaneously in doses of 0.1 cc of a 10% solution; the latter in 0.5 cc doses of a 1% solution. The results (Tables III and IV) show that adrenalin increased the resistance of adrenalectomized rats to both reagents. In the case of formaldehyde, however, the adrenalin-treated adrenalectomized rats were still more sensitive than the intact animals, while with sodium arsenite, the adrenalin-treated adrenalectomized rats were, if anything, somewhat more resistant than the intact rats.

TABLE III. Effect of Adrenalin in Formaldehyde Poisoning.

No. of inj.	5	6	7	8	9	10
No. of deaths:						
Intact			1			*
Adrenalectomized	3		1		1	
Adx. + adrenalin		1	1	1	1	1

* Of 5 intact animals, all but one were still alive after 10 injections, although they were in a state of collapse.

TABLE IV. Effect of Adrenalin in Sodium Arsenite Poisoning.

No. of inj.	3	4	5	6	7
No. of deaths:					
Intact	1	6			1
Adrenalectomized	4	3			1
Adx. + adrenalin			6	2	

Although the increased resistance in adrenalin-treated animals was always accompanied by a rise in blood sugar, the hyperglycemic

TABLE V. Comparative Effects of Adrenalin and L-arterenol.

No. of inj.	3	4	5	6	7	8	9	10	11	12	Blood sugar (terminal) mg %
No. of deaths:											
Adx. + KCl	1	6	3	2	1						105
" + adrenalin		1	2	2	2	1	1	2	1	1	185
" + L-arterenol			4	3	3		2	1			135

state appeared to be merely a measure of adrenalin activity and not the mechanism responsible for survival. The blood sugar level in the adrenalectomized rats, even terminally, was never so low as to permit an assumption that hypoglycemia was the immediate cause of death. It seemed likely that the primary effect of adrenalin was to resist circulatory collapse. Since l-arterenol (nor-epinephrine) is known to maintain vasomotion while only slightly raising the blood sugar level, the effects produced by this agent should reveal the significance of the hyperglycemia, if any. L-arterenol was therefore administered in the same manner as was adrenalin. The results (Table V) showed that adrenalin and l-arterenol had comparable effects.

It appeared, then, that the supportive effect of the medullary hormone was primarily in maintaining vascular tone, and that the blood sugar values were a rough index of the level of circulating adrenalin.

Finally, a study was made of the effects of blocking the activity of adrenalin. Two sympatholytic agents were tested: Gynergen (ergotamine tartrate) administered in doses of 0.1 cc each containing 5 γ with each injection of adrenalin; and dibenamine in doses of 0.1 cc each containing 1 mg.

It is seen from Table VI that the dose level of Gynergen used was not adequate to block the action of adrenalin or of the intact adrenal medulla. It did, however, suppress hyperglycemia to some extent. Dibenamine, on the other hand, did not affect the usual blood sugar response to adrenalin, but abolished the resistance provided by the latter.

Discussion. The findings indicate that the administration of adrenalin provides substantial protection to adrenalectomized rats against some toxic agents, and that, in the case of KCl and sodium arsenite, the prolongation of life may actually surpass that of intact rats. The production of hyperglycemia is evidently not the mechanism by which the medullary hormone participates in the resistance against these agents; animals treated with adrenalin-antagonists show enhanced susceptibility to intoxication in spite of hyperglycemia.

The relative importance of the adrenal cortex and the medulla in the maintenance of vascular tone is not established. Zweifach, *et al.*(4) obtained in adrenalectomized rats

TABLE VI. Effects of Gynergen and Dibenamine.

No. of inj.	5	6	7	8	9	10	11	12	13	14	15	16	17	Blood sugar, mg %
No. of deaths:						Adrenalectomized rats								
KCl	3		2											120
" + A*							2	1		1	1			200
" + A + G									2	2		1		170
" + A + D			2	1	1	1								230
						Intact rats								
KCl				1		1	2	1						260
" + A										1	1	1	2	360
" + G							3	1	1					190
" + D			1	1	2	1								250

* A = Adrenalin; G = Gynergen; D = Dibenamine.

restoration of the normal state in the vascular bed with cortical steroids. Works of Swingle and others(5,6) point to the role of the cortex in traumatic shock, while Huizenga, *et al.* (7) report on the ineffectiveness of cortical hormones in hemorrhagic shock. Remington (8) found that intact dogs given a large dose of dibenamine succumbed to minor hemorrhages, and that the cardiovascular changes in these animals resembled those of animals in terminal adrenal insufficiency or acute crises following bilateral adrenalectomy. Ramey, *et al.*(9) found that ACE potentiates the blood pressure response to norepinephrine in adrenalectomized dogs. We, however, were unable to confirm this in rats. Fritz and Levine(10) believe that cortical steroids are necessary for the efficient response of blood vessels to sympathin E.

Whatever may be the mechanism, the results obtained by us, together with the observations that adrenalin injection increases the resistance of adrenalectomized animals to histamine(11-13) suggest that at least under the conditions of the present experiments, the presence of the adrenal medulla is of primary importance.

Summary. 1. Administration of adrenalin prolongs survival time of adrenalectomized rats injected with KCl, formaldehyde, and sodium arsenite. 2. Under conditions of our experiments, adrenalin was more effective than cortisone in providing protection against these agents. 3. This protective effect is abolished by simultaneous administration of sympatholytic drugs. 4. The mechanism of protection appears to be through the prevention of vascular collapse. 5. It is suggested that the medullary secretion is an important factor in resisting traumatic shock.

1. Schäfer, E. A., *Brit. Med. J.*, 1908, v1, 1277, 1346.

2. Britton, S. W., and Silvette, H., *Am. J. Physiol.*, 1932, v100, 701.

3. Zwemer, R. L., and Truszkowski, R., *Endocrinol.*, 1937, v21, 40.

4. Zweifach, B. W., Schorr, E., and Black, M. M., *Ann. N. Y. Acad. Sci.*, 1953, v56, 626.

5. Swingle, W. W., and Pfiffner, J. J., *Sci.*, 1930, v72, 75.

6. Swingle, W. W., Parkins, W. M., and Remington, J. W., *Am. J. Physiol.*, 1941, v134, 503.

7. Huizenga, K. A., Brofman, B. L., and Wiggers, C. J., PROC. SOC. EXP. BIOL. AND MED., 1943, v52, 77.

8. Remington, J. W., *Am. J. Physiol.*, 1951, v165, 306.

9. Ramey, E. R., Goldstein, M. S., and Levine, R., *ibid.*, 1951, v165, 450.

10. Fritz, I., and Levine, R., *ibid.*, 1951, v165, 456.

11. Kellaway, C. H., and Cowell, S. J., *J. Physiol.*, 1922, v56, Proc. XX.

12. ———, *ibid.*, 1923, v57, 82.

13. Wyman, L. C., *Am. J. Physiol.*, 1928, v87, 29.

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Studies on Platelets. XVI. Glutamic Oxaloacetic Transaminase Activity of Human Platelets.* (22275)

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Glutamic oxaloacetic transaminase (GOT) is a specific enzyme concerned with the synthesis of glutamic and oxaloacetic acids through the transfer of α-amino nitrogen of aspartic acid to α-ketoglutaric acid. Its presence has been demonstrated in serum of many animals and man, as well as in many animal tissues(1-4). It was observed by Karmen *et al.*(4) that human blood hemolysates

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contained GO-T activity greatly in excess of that of human serum. This observation suggested the presence of GO-T in formed elements of the blood. Due to our interest in platelets, a study was done to determine the GO-T activity of these bodies.

Methods. 1) *Determination of GO-T activity* was carried out as described by Karmen *et al.*(4). A minor modification was introduced consisting in the use of 0.03 ml of malic acid reagent instead of 0.1 ml as indicated in the original technic. This modification has given results quite comparable to those obtained with the original method(5). Experiments were run in triplicate. The reacting mixture was incubated for 10′ and readings were taken at 5′-10′-15′ after addition of the aspartic acid reagent. Values given in this study are, therefore, the average of nine figures obtained for each sample. 2) *Preparation of platelet-rich and platelet-poor plasma; and of platelet suspensions in saline* was carried out as described(6). Technics differed, however, in small details. Standard ACD solution (1/5 volume) was used as anticoagulant instead of EDTA-Na_2. Blood was freshly collected from healthy donors in plastic containers and processed without delay. Volume of saline used in washing platelets was identical to that of the platelet-rich plasma originally used. Platelet suspensions were adjusted to various concentrations by addition of suitable volume of saline solution. Platelets were counted directly(7) with the use of phase microscope. All preparations were assayed for enzymatic activity within 8 hours of blood collection. 3) *Preparation of water soluble platelet extract.* This original technic is being described(8). To packed, fresh, twice washed platelets from 50 ml of fresh human blood, 6 ml of distilled water and 5 ml of precooled ethyl ether were added. After centrifugation at 3,500 rpm/30′ at 4°C, three well differentiated layers were obtained: an upper layer, containing ethyl ether and lipoid substances; a middle one containing a precipitate, probably composed of protein material and stroma, and a bottom one containing a water-soluble protein. After separation of the supernant ether layer, the middle and lower layers were brought back with distilled water to the volume of platelet-rich plasma originally used. Centrifugation at 3,200 rpm/30′ at 4°C brought to sharp separation of a top layer containing insoluble protein material and stroma from a water-clear bottom layer. The bottom layer, representing approximately 19/20 of the total volume was transferred by suction to another container, leaving behind the top layer. Enough water was then added to full reconstitution of volume.

TABLE I. Glutamic Oxaloacetic Transaminase Activity in Various Preparations from Human Platelets.*

Preparation	No. of platelets/mm^3 $\times 10^{-3}$	Units of GO-T activity
Normal plasma	235 ± 25	37.5 ± 7.5
Platelet-poor plasma	10 (approx.)	20.8 ± 2.0
Platelet suspension (saline)	2000 ±200	255.0 ±10.5
	470 ± 15	61.5 ± 3.5
	120 ± 24	43.0 ± 2.0
	40 ± 5	28.5 ± 1.5
Washings of sedimented platelets		10.5 ± 1.6
Water soluble fraction (see text)		104.2 ±10.0

* Values calculated on basis of 5 different experiments.

Results. (Table I). (a) Plasma containing an adequate number of platelets showed greater GO-T activity than platelet-poor plasma; (b) Slight GO-T activity was recovered from washing of platelets after their separation from plasma by high speed centrifugation. This may have represented GO-T activity of plasma which had remained absorbed into the platelets; (c) The transaminase activity of isolated platelet preparations containing discrete, separated bodies was in gross proportion to their number; (d) The water soluble protein obtained from platelets (bottom layer) appeared to possess high GO-T activity.

Discussion. There seems to be no question that platelets represent a source of GO-T in human blood. In view of the present uncertainty as to whether a factor discovered in platelets is an intimate constituent of these bodies or is more simply carried by them, it

became necessary to study the "location" of GO-T in human platelets. Low GO-T activity was found in washings from separated platelets. On the other hand, high activity was found in a water soluble preparation from platelets after treatment with ether. Ether appeared to destroy the physical integrity of platelets while removing most of their lipoid constituents. Both findings, therefore, suggested that GO-T was an intimate constituent of platelets, presumably a part of the protein, water-soluble fraction.

It appears premature to discuss the significance of the finding of GO-T in platelets as well as the possible relationship of the enzyme to the role of platelets in the hemostatic mechanism.

Summary. 1) Platelets are a source of glutamic oxaloacetic transaminase in human blood. 2) The enzyme is an intimate constituent of platelets, being found principally in a water soluble fraction of platelets after separation of lipoids by ethyl ether.

1. Awapara, J., *J. Biol. Chem.,* 1953, v200, 537.

2. Braunstein, A. E., *Adv. Protein Chem.,* 1947, v3, 11.

3. Awapara, J., and Seale, B., *J. Biol. Chem.,* 1952, v194, 497.

4. Karmen, A., Wróblewski, F., and LaDue, J. S., *J. Clin. Invest.,* 1955, v34, 126.

5. Mathewson, S., personal communication to authors.

6. Stefanini, M., Dameshek, W., Chatterjea, J. B., Adelson, E., and Mednicoff, I. B., *Blood,* 1953, v8, 54.

7. Quick, A. J., Shanberge, J. N., and Stefanini, M., *J. Lab. and Clin. Med.,* 1948, v33, 1424.

8. Magalini, S. E., and Stefanini, M., *Science,* in press.

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Amperometric Titration of -SH Groups in Purified Clotting Factors By Improved Method. (22276)

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In studies on specific reactive groups of proteins involved in the coagulation of blood, it was observed that compounds commonly used to alter –S–S– bonds caused inhibition of clotting activity of purified bovine prothrombin, whereas those used to alter –SH groups did not(1). No –SH groups were detected in purified prothrombin, thrombin or fibrinogen, nor during the conversion of prothrombin to thrombin. In these experiments, quantitative –SH measurements were made by the argentimetric amperometric method using an ethanol-ammoniacal titration mixture(1). Benesch, Lardy and Benesch(2) recently have emphasized that this method, as applied to protein –SH groups, is open to criticism on the grounds that the high pH of the NH_4OH-NH_4NO_3 buffer used may denature proteins and accelerate the oxidation rate of –SH groups, that the ammonium ions may effect total disappearance of some –SH groups, and that the alcohol, usually necessary to obtain sharp end points, also denatures proteins. It therefore was possible, although unlikely, that the absence of detectable –SH groups in the purified clotting products could be attributed to these factors.

In this same paper, Benesch *et al.* introduced a modification of the amperometric method which overcame these objections. Instead of ammonia as the agent to form the silver complex, they employed tris-(hydroxymethylaminomethane) which made it possible to titrate protein –SH groups in aqueous, buffered solutions at pH 7.4. Such solutions had the significant additional advantage of exerting no demonstrable drastic effects on proteins, thereby allowing the study of –SH groups of proteins in the native state and a more precise determination of the relative ac-

cessibility of these groups after treatment with denaturing agents. They further demonstrated that the Tris method is accurate, sensitive and highly specific for protein –SH groups.

Methods and materials. Several highly active samples of each of the purified prothrombin, citrate thrombin, biothrombin, autoprothrombin I and fibrinogen, all of bovine origin,* and of bovine fibrinogen (Armour and Co.) and Thrombin Topical (Parke, Davis and Co.) were titrated in triplicate for –SH groups using the Tris argentimetric amperometric method(2). The proteins were dissolved in redistilled, deionized water, as were all reagents, and they were made up to a concentration of from 5 to 10 mg/ml. Reduced glutathione was used as a standard control. Each of the proteins was titrated in its native state, and following denaturation with half saturated urea, half saturated guanidine hydrochloride and by boiling for 30 minutes, respectively.

Results. Under none of the above conditions were any –SH groups detected in the purified clotting factors, thus substantiating the results obtained indirectly with the use of –SH blocking agents and directly with the ammoniacal-ethanol amperometric method (1).

Discussion. Ethanol-ammoniacal argentimetric amperometric titrations for quantitative measurement of –S–S– bonds had been carried out on each of the above listed proteins(1,3) with results which indicated that all but fibrinogen contained 43 μM –S–S–/100 mg N; prothrombin contained 4 moles –S–S–/mole. Since the principle of the method(4) is the sulfitolysis of protein –S–S– with the release of 1 mole –SH for each mole –S–S–, the objections to the titration of –SH groups in an ethanol-ammoniacal medium were applicable to this method. Repeated attempts to adapt the Tris method to the measurement of protein –S–S– resulted in failure, even though accurate, reproducible values were achieved in the measurement of –S–S– bonds of oxidized glutathione. Although the indicated objections are valid, the extensive control studies with oxidized glutathione and with insulin, and the good agreement with the amino acid analysis of purified bovine prothrombin and of fibrinogen(1,4) would indicate that the original –S–S– values are correct, and that the "ammonium effect" is not operative with the protein products listed above.

Summary. Since the ethanol-ammoniacal amperometric method for quantitative measurement of –SH groups in proteins is susceptible to valid criticism, measurements were repeated on certain clotting proteins using the Tris buffer system. This procedure also resulted in the inability to detect –SH groups in these proteins.

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1. Carter, J. R., and Warner, E. D., *Am. J. Physiol.,* 1954, v179, 549.

2. Benesch, R. E., Lardy, H. A., and Benesch, R., *J. Biol. Chem.,* 1955, v216, 663.

3. Carter, J. R., and Warner, E. D., *Am. J. Physiol.,* 1956, v184, 195.

4. Carter, J. R., *Science,* 1954, v120, 895.

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Acetal Phosphatides in the Adipose Tissue of Newborn Rats. (22277)

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Since the discovery of acetal phosphatides by Feulgen and Voit(1) a considerable amount of work has been done upon the distribution and isolation of acetal phosphatides in tissues. Few investigators have made quantitative chemical studies of the metabolic role of these compounds. Stetten and Schoenheimer(2), basing their conclusions upon experiments utilizing deuterium labeled compounds, have indicated that fatty aldehydes may be intermediates in the interconversion of fatty acids and fatty alcohols. On the other hand, Ehrlich and Waelsch(3), also using deuterium labeled fatty acids, concluded that fatty aldehydes were not directly involved in the overall synthesis or transport of fatty acids. The suggestion that acetal phosphatides are involved in both the anabolism and catabolism of fatty acids has been made by Danielli(4), Fawcett(5), Schäfer (6) and Möckel(7). Möckel, basing his conclusions upon a series of histological studies of the adipose tissues of newborn rats and of rats subjected to starvation, proposed that the anabolism and catabolism of neutral fat follows the pathway of fatty aldehyde to acetal phosphatide.

Because of the need for quantitative chemical data on the changes in acetal phosphatide content of tissues actively engaged in lipid metabolism it was decided to investigate the changes in acetal phosphatide in relation to phospholipid and total lipids in the adipose tissues and livers of newborn rats over the first 21 days of life.

Materials and methods. Newborn rats at 1, 2, 3, 5, 8, 15 and 21 days of age were sacrificed by decapitation. The livers and samples of subcutaneous adipose tissue from the pectoral and pelvic regions were removed and freed from foreign tissue. In age groups 1, 2, 3, 5 and 8 days, tissues of 2 or 3 animals were pooled. In pooling tissues, care was taken to assure that the number of animals taken from any one litter was the same for each age group. The lipid of the tissues was extracted by the method of Rice and coworkers(8). The extracts were then analyzed for acetal phosphatide, total phospholipid and total lipid. Acetal phosphatides were determined by the method of Feulgen, Boguth and Andresen(9). Phospholipid phosphorus was determined by the procedure of Fiske and Subbarow(10). Total lipids were determined gravimetrically.

Results. The results are summarized in Table I. This table represents the average results from 2 separate series of experiments. In each series the changes noted were parallel and of the same order of magnitude. Preliminary experiments where only acetal phosphatides were determined had shown that

TABLE I. Lipid Changes in Adipose Tissues and Livers of Rat during First 3 Weeks of Life.*

	Adipose tissue			Liver		
Age, days	Total lipid, %	Phospholipid phosphorus, μmoles/g	Acetal phosphatide, μmoles/g	Total lipid, %	Phospholipid phosphorus, μmoles/g	Acetal phosphatide, μmoles/g
1	19.1	28.10	1.72	7.31	25.2	.401
2	29.0	25.40	1.02	4.10	19.5	.508
3	42.5	17.90	.61	4.67	18.8	.392
5	45.8	1.90	.21	5.43	27.3	.406
8	61.5	1.24	.05	5.41	28.7	.383
15	59.1	3.37	.08	4.63	31.2	.472
21	68.9	1.71	.03	4.98	36.1	.307

* All values in terms of wet wt of tissue.

there was some variation among litters in values obtained from animals at the first 3 age groups. This variation was associated with the number of animals in the litter, since animals from the larger litter showed a higher initial acetal phosphatide content in the adipose tissue. The changes associated with increased age for each litter were parallel and of the same order of magnitude. In the experiment described above and where tissues were pooled, the variation among litters was minimized by keeping the number of animals from any one litter the same for each age group.

Discussion. The changes in the lipid fractions of the liver with increased age in newborn rats were within normal adult limits and little significance can be attributed to them. These data, however, do not exclude the possibility that acetal phosphatides may be involved in the lipid metabolism of the liver by means of a turnover mechanism. In adipose tissue, on the other hand, the maximum changes occurred during the first 5 days of life. The initially high levels of acetal phosphatide and total phospholipid decreased rapidly to only trace amounts as the adipose tissue filled with fat. The results confirm and extend the observation of Möckel(7). The inverse changes in acetal phosphatide and phospholipid, as compared to total lipid, indicate that they are in some way involved in the laying down of fat in adipose tissue. Acetal phosphatides could be involved directly in this mechanism or they could be involved in the synthesis of other phospholipids which could then play some role in the fat metabolism of adipose tissue.

Summary. The changes in the acetal phosphatide, phospholipid and total lipids have been followed in the adipose tissues and livers of newborn rats over the first 3 weeks of life. As the adipose tissue fills with fat, the acetal phosphatide and total phospholipid, initially quite elevated, decrease rapidly to only trace amounts by the fifth to eighth day of life. These results indicate that acetal phosphatides and phospholipids are involved in some manner in the laying down of fat in adipose tissue.

1. Feulgen, R., and Voit, K., *Arch. ges. Physiol.*, 1924, v206, 389.

2. Stetten, D., and Schoenheimer, R., *J. Biol. Chem.*, 1940, v133, 347.

3. Ehrlich, G., and Waelsch, H., *ibid.*, 1946, v163, 195.

4. Danielli, J. F., *Quart. J. Micr. Sci.*, 1950, v91, 215.

5. Fawcett, D. W., *J. Morph.*, 1952, v90, 363.

6. Schäfer, G., *Arztl. Forsch.*, 1950, v4, 46.

7. Möckel, G., *Z. physiol. Chem.*, 1943, v277, 135.

8. Rice, L. I., Shotz, M. C., Alfin-Slater, R. B., and Deuel, H. J., *J. Biol. Chem.*, 1953, v201, 867.

9. Feulgen, R., Boguth, W., and Andresen, G, *Z. physiol. Chem.*, 1951, v287, 90.

10. Fiske, C. H., and Subbarow, Y., *J. Biol. Chem.*, 1925, v66, 375.

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Effect of Para-Aminosalicylic Acid on Serum Isoniazid Levels in Man. (22278)

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Previous investigations(1,2) with chemical methods have shown wide variations in the rate of metabolic alteration of isoniazid (INH) from person to person, but a relatively constant rate in any one individual. This observation is of clinical importance because only the free, unaltered isoniazid has significant antimicrobial activity against tubercle bacilli(3). One of the major pathways of metabolic disposition of INH is by acetylation(1). *In vitro* experiments by Johnson (4) have shown that para-aminosalicylic acid (PAS) inhibits the acetylation of INH by a competitive mechanism.

The purpose of this report is to present observations on the application of a specific microbiologic assay technic to determine the effect of PAS on the level of antimicrobially active INH in human subjects.

Materials and methods. The technic of microbiologic assay for INH was as follows: 1 ml of serum from aseptically collected specimen of clotted venous blood was added to 9 ml of oleic acid-albumin medium ST-T* containing 10 μg PABA/ml. From this tube of 1:10 serum dilution, 2.5 ml aliquots of serum dilutions of 1:10, 1:20 and 1:40 were prepared in duplicate in ST-T medium in screw-capped culture tubes. Each of 6 tubes for any one determination was then inoculated with 0.1 ml of fully-grown, dispersed, stock culture of an INH-susceptible, streptomycin-resistant strain of tubercle bacilli (H37RvS-RSM) grown in ST-T medium containing 0.05% Tween 80 and 10 μg streptomycin/ml. The inoculated tubes, along with standard control test of susceptibility of the strain to known concentrations of INH (0, .02, .04, and .08 μg INH/ml), were incubated at 36°. After 5 days, a Ziehl-Neelsen stained smear from each tube was prepared and the tubercle bacilli examined for loss of acid-fastness (AF). This is a specific test for antimicrobially active INH as neither human sera nor any known antimicrobial agent other than INH has this effect on tubercle bacilli(6). The results were read as follows: (1) No loss of AF at 1:10 dilution (<0.3 μg INH/ml); (2) Moderate loss of AF at 1:10 dilution, no loss at 1:20 (0.3 μg INH/ml); (3) Marked loss of AF at 1:10, no loss at 1:20 (0.4-0.6 μg INH/ml); (4) Moderate loss of AF at 1:20, no loss at 1:40 (0.7 μg INH/ml); (5) Marked loss of AF at 1:20, no loss at 1:40 (0.8-1.4 μg INH/ml); (6) Moderate loss of AF at 1:40 (1.5 μg INH/ml); (7) Marked loss of AF at 1:40 (1.6 μg or more INH/ml). Preliminary studies with known amounts of INH added to human sera permit assignment of these values for INH equivalents in μg/ml. It was also shown that addition of as much as 100 μg streptomycin/ml and 1000 μg PAS/ml to undiluted human sera did not interfere with this microbiologic assay. A total of 76 assays were performed on 25 patients under treatment for tuberculosis. Patients were selected from a group who did not maintain serum INH levels in excess of 0.3 μg/ml 6 hours after oral loading dose of 4 mg INH/kg in tablet form (Nydrazid, Squibb). This standard loading dose regimen required that

TABLE I. Effect of PAS on Microbiologic Assay of Serum INH, in 25 Patients.

	Loading dose, mg/kg	Without PAS, μg INH/ml	With PAS, μg INH/ml	Result*
H.W.	4	<.3	.8–1.4	+
T.H.	"	"	.4– .6	"
C.H.	"	"	.8–1.4	"
W.C.	"	"	.4– .6	"
W.D.	"	"	"	"
W.S.	"	"	.3	"
P.M.	"	"	.4– .6	"
G.R.	"	"	<.3	0
M.P.	"	"	"	"
C.M.	8	"	.8–1.4	+
E.R.	"	.4– .6	"	"
M.B.	"	"	"	"
A.B.	4	<.3	.4– .6	"
	"	.3	"	"
S.P.	"	<.3	.8–1.4	"
	"	"	.3	"
M.H.	8	"	.4– .6	"
	"	"	.8–1.4	"
F.H.	"	.4– .6	"	"
	"	"	"	"
B.S.	4	<.3	.7	"
	8	.3	.8–1.4	"
J.E.	4	<.3	<.3	0
	8	"	.4– .6	+
S.C.	4	"	"	"
	8	.4– .6	.8–1.4	"
A.J.	4	<.3	.4– .6	"
	8	.4– .6	.8–1.4	"
B.Y.	4	<.3	.4– .6	"
	8	.3	"	"
R.C.	4	<.3	<.3	0
	8	.4– .6	.8–1.4	+
M.B.	4	<.3	.4– .6	"
	8	.8–1.4	.8–1.4	0
M.S.	4	<.3	<.3	"
	8	.3	.4– .6	+
M.G.	4	<.3	<.3	0
	8	"	"	"

* + = Increase; 0 = No detectable change.

* Same as "basic medium" previously described(5) except for addition of 0.4 g sodium citrate, 0.01 g ferric ammonium citrate and 2 g glycerol/l; 0.5% oleic acid-albumin complex plus 0.2% glucose (final concentrations) were added aseptically after autoclaving basic medium.

the patient have received total daily dose of INH from 8 to 16 mg/kg for at least one day prior to test and that, except for streptomycin, he receive no antimicrobial agent other than INH. The INH was given at 9 a. m. and blood was collected at 3 p. m. When PAS (Hellwig, PAS Sodium tablets) was administered, it was given in a total daily dose of 10 g of free PAS, 2.5 g at 9 a. m., 12 p. m., 4 p. m. and 8 p. m. Each study was performed under the same conditions with or without PAS which was given or withheld for 5 days prior to each determination.

Results. The results are listed in Table I. Twenty-two of the 25 patients showed a detectable increase in their microbiologically active serum levels of INH when PAS was also given. Repeat determinations with the same loading dose of INH were performed on 4 patients. They showed increases each time that PAS also was administered. Repeat determinations with different loading doses of INH were performed on 9 patients. Five were done because there was no detectable change in the first study. In 4 of these 5 subjects there was a detectable increase in INH level when PAS was added. The absence of a detectable increase in 3 of the 25 patients studied did not necessarily mean that PAS had no effect in these individuals for the minimum serum dilution employed (1:10) did not permit detection of possible changes below 0.3 μg INH/ml.

Discussion. The biologic phenomenon of one drug raising the blood level of another drug is not uncommon. The results of the present study demonstrate that concurrent administration of PAS with INH raises the serum concentration of INH when measured 6 hours after administration of INH. In view of Johnson's observation(4), it appears probable that PAS achieves this effect by decreasing the rate of acetylation of INH to the antimicrobially inactive acetyl derivative.

It seems fair to assume that delivery of effective concentrations of INH to multiplying populations of tubercle bacilli in tuberculous patients is determined in part by the concentration of INH in the circulating blood. Therefore, the problem of adequate dosage of INH presents itself in those tuberculous patients who dispose of INH so rapidly that effective antimicrobial levels cannot be achieved or maintained. The present studies show that these levels can be elevated by increasing the dosage of INH or by adding PAS.

The therapeutic superiority of treatment with INH plus PAS over INH alone in human beings(7) may be attributable not only to the antimicrobial activity of PAS itself, but also to the delivery of higher concentrations of INH to multiplying tubercle bacilli.

Summary. A method for the microbiologic assay of INH in the presence of high concentrations of streptomycin or PAS is described. The concurrent oral administration of PAS with INH resulted in detectable elevation of the antimicrobially active INH level in the blood serum of 22 of 25 tuberculous patients. The significance of this effect is discussed.

1. Hughes, H. B., Schmidt, L. H., and Biehl, J. P., *Trans. of 14th Conf. on Chemotherapy of Tuberculosis* (Veterans Admin., Army, Navy,), 1955, 217.

2. Cuthbertson, W. F. J., Ireland, D. M., Wolff, W., and Kuper, S. W. A., *Brit. Med. J.*, 1954, v70, 266.

3. Hughes, H. B., Biehl, J. P., Jones, A. P., and Schmidt, L. H., *Am. Rev. Tuberc.*, 1954, v70, 266.

4. Johnson, W. J., *Nature*, 1954, v174, 744.

5. Cohn, M. L., Oda, U., Kovitz, C., and Middlebrook, G., *Am. Rev. Tuberc.*, 1954, v70, 465.

6. Middlebrook, G., *ibid.*, 1952, v65, 765.

7. Eckley, G., *Trans. of 13th Conf. on Chemotherapy of Tuberculosis* (Veterans Admin., Army, Navy), 1954, 134.

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Survival of *Histoplasma capsulatum* in Experimental Histoplasmosis in Mice.* (22279)

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Previous studies(1,2) have demonstrated that fatal infections following intraperitoneal inoculation of white mice with yeast phase *H. capsulatum* occur within a 30-day period. After this "acute" period very few of the survivors died within the ensuing year. This study was conducted to determine the fate of *H. capsulatum* after inoculation into white mice.

Materials and methods. Three groups of white Swiss male mice were used in these studies. The intraperitoneal route of inoculation was used throughout. At 8 weeks of age one group received 7 million heat-killed cells, while a second group was inoculated with 0.7 million live yeast-phase organisms in 0.5 ml of mucin, as previously described(2). Following a 6-week period, the "immunized" group and survivors of the second group were challenged with 35 million organisms as was a third control group of 14-week-old mice. At various intervals up to 53 weeks after infection, 8 to 14 mice were sacrificed and cultures obtained. From each animal the whole spleen, heart, right kidney and right lung, and a 1 cm portion of liver were removed, aseptically. Each organ was ground in a sterile mortar containing 2 ml of sterile physiologic saline, after which 1 ml of the ground tissue was inoculated on each of 2 Sabouraud's agar plates containing 80 units and 0.5 mg per ml each of penicillin and streptomycin, respectively. The plates were sealed with parafilm, incubated at room temperature, and read at 15 and 30 days. Those cultures showing a solid growth of *H. capsulatum* were arbitrarily designated 4^+, heavy but not solid growth as 3^+, more than 40 colonies as 2^+ and less than 40 as 1^+. The sum of the concentrations divided by the number of plates counted was designated as the ARC (average relative concentration of organisms).

Results. Table I shows the results obtained in mice receiving a single challenge dose of 35 million organisms (Group I). During the first 5 weeks all of 14 mice showed positive cultures from the lungs, liver and spleen, while the heart's blood and kidney yielded 11 and 10 positive cultures, respectively. A relatively higher concentration of organisms was observed in the spleen (2.7), liver (2.4) and kidney (2.0) than in the lung (1.7) and heart's blood (1.5). Of 12 mice sacrificed between 6 to 10 weeks after inoculation, positive cultures were obtained from the spleen and liver in 11 and the kidney in 10, while the heart and lungs yielded only 5 and 2 positive cultures, respectively; the average concentration of organisms was likewise higher in the reticuloendothelial tissues (2.6 to 3.2) as compared to that observed in the heart's blood (1.3) and lung (1.0). A similar relationship was observed in 16 mice between 11 to 15 weeks except for only 5 positive cultures from the kidney as compared to 12 and 14 from the spleen and liver, respectively. After 16 weeks no organisms were recovered from the lungs of 38 mice studied while only 1 showed a positive heart's blood culture. This was in contrast to the kidneys, liver and spleen which yielded 42.8, 21.4 and 35.7% positive cultures, respectively, between 16 to 30 weeks, and 35.7, 21.4 and 14.3%, respectively, between 31 to 45 weeks. During the 46- to 53-week period positive cultures were obtained only from the spleen and kidney of 1 of the 10 mice necropsied.

The results in mice previously "immunized" with 7 million heat-killed cells (Group II) were essentially similar as can be seen in Table I. Again it was noted that after 16 weeks the lungs and heart's blood had been cleared of the organisms. The only suggested difference was the relative decrease of the average concentration of organisms in the

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TABLE I. Survival of *H. capsulatum* in Mice following Primary Challenge, Reinfection, and after "Immunization."

Tissue	Wk post-inoc.	Positive cultures: Group I* No.†	%	ARC‡	Group II* No.	%	ARC	Group III* No.	%	ARC
Heart	1– 5	11/14	78.6	1.5	6/8	75.	1.7	13/15	86.7	2.1
	6–10	5/12	41.7	1.3	3/8	37.5	1.3	6/12	50.	1.7
	11–15	2/16	12.5	1.0	1/8	12.5	0	0/ 5	0	0
	16–30	0/14	0	0	0/9	0	"	1/ 7	14.3	1.0
	31–45	1/14	7.1	1.0	0/8	"	"	—	—	—
	46–53	0/10	0	0	—	—	—	—	—	—
Lung	1– 5	14/14	100.	1.7	7/8	87.5	1.9	13/15	86.7	2.0
	6–10	2/12	16.7	1.0	2/8	25.	1.5	6/12	50.	1.7
	11–15	5/16	31.3	"	"	"	1.0	2/ 5	40.	1.0
	16–30	0/14	0	0	0/9	0	0	1/ 7	14.3	"
	31–45	"	"	"	0/8	"	"	—	—	—
	46–53	0/10	"	"	—	—	—	—	—	—
Kidney	1– 5	10/14	71.4	2.0	7/8	87.5	2.0	14/15	93.3	1.6
	6–10	10/12	83.3	3.1	6/8	75.	2.3	9/12	75.	1.9
	11–15	5/16	31.3	2.0	2/8	25.	1.5	3/ 5	60.	2.5
	16–30	6/14	42.8	1.7	1/9	11.1	1.0	3/ 7	42.8	2.0
	31–45	5/14	35.7	1.8	2/8	25.	1.5	—	—	—
	46–53	1/10	10.	1.0	—	—	—	—	—	—
Liver	1– 5	14/14	100.	2.4	8/8	100.	3.0	14/15	93.3	3.4
	6–10	11/12	91.7	2.6	7/8	87.5	1.9	10/12	83.3	3.0
	11–15	14/16	87.5	1.6	4/8	50.	1.3	3/ 5	60.	1.5
	16–30	3/14	21.4	"	2/9	22.2	1.0	3/ 7	42.8	1.3
	31–45	"	"	1.3	1/8	12.5	"	—	—	—
	46–53	0/10	0	0	—	—	—	—	—	—
Spleen	1– 5	14/14	100.	2.7	8/8	100.	3.3	15/15	100.	3.3
	6–10	11/12	91.7	3.2	"	"	2.5	11/12	91.7	2.5
	11–15	12/16	75.	1.8	5/8	62.5	1.8	3/ 5	60.	1.5
	16–30	5/14	35.7	1.6	3/9	33.3	1.7	5/ 7	71.4	2.0
	31–45	2/14	14.3	1.0	1/8	12.5	1.0	—	—	—
	46–53	1/10	10.	1.0	—	—	—	—	—	—

* I = Primary challenge with 35 million organisms. II = Challenge 6 wk after dose of 7 million heat-killed organisms. III = Challenge 6 wk after dose of 0.7 million organisms.
† Numerator = No. positive cultures. Denominator = Total No. cultured.
‡ Avg relative concentration of organisms (see text).

liver (3 to 1.9) and spleen (3.3 to 2.5) between 6 to 10 weeks, as compared to that observed between 1 to 5 weeks; this observation was in contrast to the results noted in Group I where average concentrations in the liver rose slightly from 2.4 to 2.6, and in the spleen from 2.7 to 3.2 (Table I).

Similar results were obtained in the mice which previously had survived sublethal infections with 0.7 million organisms (Group III, Table I). Here again as in Group II there seemed to be the trend downward at 6 to 10 weeks as compared to 1 to 5 weeks in the average concentration of organisms in both the liver and spleen from 3.4 to 3.0 and 3.3 to 2.5, respectively. In this group which had received 2 inoculations of viable organisms, the liver and spleen had 42.8 and 71.4% positive cultures, respectively, between 16 to 30 weeks. During this same time interval the liver had about 22% and the spleen about 35% positive cultures in both Groups I and II.

Discussion. Mortalities in white mice following intraperitoneal inoculation of yeast phase *H. capsulatum* in mucin usually occur within 30 days(1,2). Although deaths from specific infection rarely occur after this period it is evident that organisms are still present in the heart's blood and lungs as long as 11 to 15 weeks after inoculation. The spleen, liver and kidney show positive cultures as late as 45 weeks post-inoculation. Relatively little difference was noted between mice receiving primary challenge (Group I) as compared to those receiving heat-killed organisms

(Group II) or sub-lethal doses (Group III) 6 weeks prior to challenge. The only suggested differences were: 1) an almost 2-fold greater number of positive liver and spleen cultures between 16 to 30 weeks in Group III as compared to the other 2 groups; this could probably be attributed to the residual viable organisms from the inoculation made 6 weeks prior to challenge. 2) A relatively higher concentration of organisms in the liver and spleen between 6 to 10 weeks in Group I as compared to the other 2 groups. When compared to the concentrations of organisms observed between 1 to 5 weeks, there was a definite trend downward in the previously infected or "immunized" mice as compared to the relative rise at 6 to 10 weeks in the primary challenge group.

Summary. Yeast phase *H. capsulatum* when inoculated, intraperitoneally, into white Swiss male mice may be recovered frequently from reticuloendothelial tissues as late as 45 weeks after infection. The lungs and heart's blood usually clear within 16 weeks post-inoculation. Mice surviving previous sub-lethal challenge or those previously given 1 dose of heat-killed organisms show little variation in these respects, when challenged, from those receiving primary challenge.

1. Schaefer, J., and Saslaw, S., PROC. SOC. EXP. BIOL. AND MED., 1954, v85, 223.

2. Campbell, C. C., and Saslaw, S., *Pub. Health Rep.*, 1951, v66, 570.

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Inhibition of Immunological Enhancement by Endotoxin in Refractory Rabbits. Immunochemical Study.* (22280)

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When Gram negative bacterial endotoxins are repeatedly administered by vein over short periods of time, resistance to the reactions normally produced by these toxic complexes rapidly develops(1). Quantities of endotoxin originally productive of vigorous systemic adjustments no longer produce untoward reactions(2,3). For example, febrile responses normally produced are either obliterated completely(2) or modified. Hemorrhagic and necrotizing phenomena normally produced in tumor tissue and skin prepared for the local Shwartzman reaction do not occur(3,4) after intravenous injection of toxin in refractory rabbits. The generalized Shwartzman reaction is no longer produced by paired spaced intravenous injections of toxin, and the leukopenia producing action of endotoxin is obliterated(5). In addition, refractoriness to the lethal effect of endotoxins occurs(6). Refractoriness to most of these reactions develops very soon after intravenous injections of endotoxin are begun. Resistance to the lethal effect of toxin may be demonstrated as early as 24 hours following the first intravenous injection(7). Refractoriness to each of the other effects of endotoxin occurs within a few days(2-4). This development has been dissociated from the classical immune response primarily by its lack of specificity(8), rapidity of development(7), shortness of duration(2), and lack of correlation with appearance and level of circulating antibody(9,10). Further, agammaglobulinemic patients unable to produce demonstrable circulating antibody develop refractoriness to febrile and intoxicating actions of endotoxins just as readily as do normal persons(11). On

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the other hand, refractoriness is promptly reversed following intravenous injection of certain colloidal substances(8,12), an effect attributed to the capacity of these colloidal substances to produce "blockade" of the reticuloendothelial system(13). Our recent studies have shown that the reactions produced by administration of Gram negative bacterial endotoxins result in profound disturbances in defense mechanisms of the host(14). Meningococcal endotoxin in quantities which prepare rabbits for the generalized Shwartzman phenomenon decreases the ability of rabbits to clear colloidal materials from the blood presumably by interfering with the function of the reticuloendothelial system(13). Further, endotoxin seemed to enhance certain bacterial infections in rabbits. For example, when toxin was given prior to establishment of a pneumococcal skin infection, bacteremia and death resulted whereas in control rabbits given no endotoxin the organism produced only local skin infection(14). In contradistinction, endotoxin did not enhance susceptibility to infection when injected intravenously in rabbits previously made refractory to the endotoxin(14). Indeed, refractory rabbits appeared to resist pneumococcal and streptococcal infection more vigorously than did controls. These observations provoked investigation of the effect of meningococcal endotoxin on the immune response in rabbits as revealed by immunochemical study. In confirmation of observations of Johnson *et al.* (15) we found that when toxin was given together with bovine serum albumin, enhancement of antibody production against the foreign serum protein resulted(16).

The present study was undertaken to determine whether refractoriness to the febrile, toxic, and necrotizing effects of endotoxin would interfere with the adjuvant effect of this material on the immune response.

Materials and methods. Animals employed were 2 kg albino rabbits obtained from one breeder and maintained throughout the experiment on a diet of Purina rabbit pellets. Endotoxin was prepared from lots of a culture-free meningococcal‡ filtrate of high Shwartzman activity. Different lots were combined, spun at 15,000 rpm, the supernatant quick-frozen and stored at –30°C. Details of preparation have been described previously(17). The refractory state was induced using a regimen which had proved to be effective in quickly producing a high degree of resistance in a short time, including refractoriness to fever, lethal effect, necrotizing action and production of leukopenia. This schedule consisted of 11 daily intravenous injections of 2 cc of endotoxin in increasing amounts, beginning with a dilution of 1:10,000 and extending to a dilution of 1:10 on the 11th day. Toxin solutions were prepared daily from the frozen culture free filtrates and dilutions made up with pyrogen free saline. Attempt to provoke the Shwartzman reaction was used as the test for resistance since it had previously been shown that this was a delicate index of susceptibility to the action of endotoxin. This reaction was ordinarily produced by an intradermal preparatory injection of 0.25 ml of 1:2 dilution of endotoxin, followed in 18 hours by an intravenous injection of the same material. In the stock of rabbits used and with the endotoxin employed, the Shwartzman reaction developed in an incidence between 95 and 100%. All injections, with the exception of the intradermal preparation for the local Shwartzman reaction, were made by way of the marginal ear vein. Crystallized bovine serum albumin (BSA), lot #67009 (Armour and Co., Chicago, Ill.), was employed as the antigen. Immediately prior to injection, solutions containing 15 mg BSA were made up to 2 ml with either pyrogen free saline or 1:100 dilution of the endotoxin depending upon the experimental group. Prior experiments established that this quantity of toxin represents the optimal dose for producing enhancement of antibody formation with the quantities of antigen used. Rabbits were divided into 3 groups; Group I normal, non-refractory rabbits given BSA together with endotoxin intravenously, Group II refractory rabbits given endotoxin and BSA antigen intra-

‡ Strain 44-B, generously supplied by Dr. Gregory Shwartzman; Mount Sinai Hospital, New York.

venously, and Group III normal rabbits given BSA intravenously.

Blood was collected by cardiac puncture on the 11th day following antigenic stimulation, since previous studies had established that at this time circulating antibody to BSA is at its highest level. Clots were broken up after standing 24 hours at 4°C, the tubes centrifuged for 20 minutes at 1000 RPM, the serum decanted into lusteroid tubes, frozen at –70°C, and stored at –30°C until antibody determinations were performed.

Serum antibody content was determined using a modification of the quantitative precipitin method described by Kabat and Mayer(18). The method involves two precipitations. The first, a preliminary run, requires only small quantities of serum and is used primarily to determine the optimal antigen concentration to be used in the final precipitation. With the preliminary precipitation, a surprisingly accurate estimate of antibody content can be made merely by analysis of the supernatant for the presence of excess antigen. Known concentrations of antigen were prepared from standard stock solution of BSA prior to each precipitation. Serum samples were thawed at 37°C for 10 minutes, mixed and centrifuged in the cold (4°C) for 1 hour at 3000 rpm. Aliquots of each serum sample were then precipitated with 4 concentrations of antigen prepared from the standard BSA solution. Precipitin tubes were incubated at 37°C for 1 hour and stored in the cold 7 days to insure complete precipitation with agitation each day. Tubes were then centrifuged at 4°C for 1 hour at 3000 rpm and supernates saved for analysis. Excess antigen was determined by capillary tube precipitation with a potent anti-BSA rabbit serum. The tubes were incubated 1 hour at 37°C, placed in the cold and read for precipitate at 36 hours. Final determinations were performed in a similar manner except that nitrogen content of the precipitates was determined. Precipitation was carried out with antigen concentrations known to fall in the equivalence zone of each serum sample. Precipitates were washed twice with 0.145 molar saline (4°C) and precipitate nitrogen was determined colorimetrically using Heidelberger MacPherson modification of the Folin-Ciocalteau reaction(19). Optical density, read at 650 mμ on a Coleman Universal Spec-

TABLE I.

Group	Experimental procedure	Serum antibody content γ N/ml	t	P
I	2 ml I.V. solution containing 15 mg BSA + 1:100 dilution of meningococcal endotoxin	333		
		201		
		74		
		121		
		94		
		233		
	Mean	176		
II	Resistant rabbits, 2 ml I.V. solution containing 15 mg BSA + 1:100 dilution meningococcal endotoxin	45		
		58		
		<10		
		<10		
		58		
		<10		
	Mean	31.8		
III	Controls—2 ml I.V. solution containing 15 mg BSA only	47		
		<10		
		<10		
		28		
		35		
	Mean	26.0		
I & II			3.47	<.02
I & III			3.65	"
II & III			.47	>.50

trophotometer Model 14, was converted to mg nitrogen using a standard calibration curve prepared with a highly purified human gamma globulin.§ Nitrogen content of the stock solution was determined by the micro-Kjeldahl method.

Results. Resistance was developed to a high degree in rabbits receiving daily intravenous injections of meningococcal endotoxin. Six of the 7 rabbits of Group II showed no evidence of a positive local Shwartzman reaction. Lack of usual febrile responses to a standardized pyrogenic dose of endotoxin was characteristic of the resistant rabbit.

The results of the quantitative precipitin determinations are shown in Table I. When meningococcal endotoxin was given together with BSA, a significant enhancement of antibody formation resulted. The mean level of circulating antibody to the BSA on the 11th day following antigenic stimulation was 6 times that of the control animals of Group III which received BSA alone. The probability that this might result from chance distribution is <0.02 which indicates that there exists a highly significant difference between these groups. The quantity of endotoxin employed, 2 ml of 1:100 dilution, was in that range recently reported to be optimal for producing an adjuvant effect resulting in increased levels of circulating antibody against apparently unrelated foreign serum protein(16).

Resistant animals of Group II received the same antigenic stimulation that resulted in greatly elevated levels of circulating antibody in the rabbits of Group I. However, serum antibody levels of the resistant animals on the 11th day following antigenic stimulation were not elevated. The difference between Groups I and II were also significant while statistical analysis revealed no difference between Groups II and III.

Discussion. It is shown in these experiments that resistance to the adjuvant effect of endotoxin was readily induced by repeated daily intravenous injections of increasing amounts of this material. A solution consisting of bovine serum albumin and meningococcal endotoxin produced significantly more antibody when given to normal untreated rabbits then when injected intravenously into refractory animals.

In these experiments the capacity of endotoxin to enhance antibody production is again demonstrated and it is further shown that this effect is prevented by prior treatment of rabbits with endotoxin according to a regimen designed to produce refractoriness to the other known effects of the toxin. These observations provide evidence that the enhancing effect of meningococcal endotoxin on the production of immune bodies cannot be explained simply as the synergistic effect of two unrelated antigens or by an unsuspected antigenic relationship between BSA and meningococcal endotoxin resulting in enhanced antibody production against the former.

It appears that expression of the "toxic" effect of endotoxin is necessary for the stimulation of antibody production by endotoxin observed in this and previous studies(15,16). The nature of this adjuvant effect remains enigmatic. Such an action could be directed specifically at the mechanism of antibody synthesis at a cellular or subcellular level or might act indirectly by mechanisms involving alteration in vascular or cellular permeability, circulatory dynamics, phagocytic function or central nervous system function. Whatever be the mode of action, the adjuvant effect of endotoxin appears to be real and to depend on the "toxic" properties of the bacterial product.

Summary and conclusion. 1. Using an immunochemical system, inhibition of the adjuvant effect of endotoxin by development of refractoriness to the "toxic" properties of endotoxin is demonstrated. 2. The basis for enhancement of immune body formation by endotoxin is discussed.

§ Generously supplied by Dr. John T. Edsall, Harvard University, Boston.

1. Thomas, L., *Ann. Rev. Physiol.,* 1954, v16, 467.
2. Beeson, P. B., *J. Exp. Med.,* 1947, v86, 29.
3. Cluff, L. E., and Bennett, I. C., Jr., PROC. SOC. EXP. BIOL. AND MED., 1951, v77, 461.
4. Shear, M. J., *J. Nat. Cancer Inst.,* 1943-44, v4, 461.

5. Unpublished observations.

6. Zahl, P. A., and Hunter, S. H., PROC. SOC. EXP. BIOL. AND MED., 1943, v52, 116.

7. Orskov, J., and Kauffman, F., *Z. F. Hyg.*, 1936, v119, 65.

8. Beeson, P. B., *J. Exp. Med.*, 1947, v86, 39.

9. Morgan, H. R., *J. Immunol.*, 1948, v59, 129.

10. Favorite, G. O., and Morgan, H. R., *J. Clin. Invest.*, 1947, v21, 589.

11. Good, R. A., *Journal-Lancet*, 1955, v75, 245.

12. Bennett, I. C., Jr., PROC. SOC. EXP. BIOL. AND MED., 1952, v81, 248.

13. Cornwell, S., and Good, R. A., *Anat. Rec.*, 1953, v115, 295.

14. Condie, R. M., Zak, S. J., and Good, R. A., *Fed. Proc.*, 1955, v14.

15. Johnson, A. G., Haines, S., and Landy, M., *ibid*, 1954, v13, 499.

16. Condie, R. M., Zak, S. J., and Good, R. A., PROC. SOC. EXP. BIOL. AND MED., 1955, v90, 355.

17. Thomas, L., and Good, R. A., *J. Exp. Med.*, 1952, v96, 605.

18. Kabat, E. A., and Mayer, M. M., *Experimental Immunochemistry*, Charles C. Thomas, Springfield, 1948.

19. Heidelberger, M., and MacPherson, C. E. C., *Science*, 1943, v98, 63.

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Progestational Effectiveness of 19-nor-Ethinyl-Testosterone* by Oral Route in Women. (22281)

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Tullner and Hertz(1) have previously demonstrated that 19-nor-progesterone is 4 to 8 times as active as progesterone by injection in the Clauberg rabbit test. Hertz, Tullner, and Raffelt(2) also showed that 19-nor-ethinyl-testosterone is likewise about 5 times as active as ethinyl testosterone when tested for progestational activity by oral administration in the Clauberg rabbit and that it is also highly active in the ovariectomized monkey pretreated with estrogen(2,3).

This report presents observations on the progestational effectiveness of 19-nor-ethinyl-testosterone† upon oral administration in 4 women.

Case 1: S.J.M. 14-year-old white female; proven by biopsy of ovarian anlagen to have ovarian agenesis (October, 1953). She was maintained on cyclic estrogen therapy with monthly withdrawal bleeding until January, 1954. Then after 23 days on 0.1 mg ethinyl estradiol daily she received 10 mg 19-nor-ethinyl-testosterone† twice daily for 12 days. Suction curettement of the endometrium showed marked progestational effects with numerous polyhedral as well as spindle cells in the stroma. Menses ensued 2 days after cessation of therapy.

Case 2: A. H. 21-year-old white female; ovarian agenesis proven by biopsy of both ovarian anlagen in November, 1953. She was maintained on cyclic estrogen therapy until January, 1954, when she was given 5 mg of 19-nor-ethisterone twice daily in addition to her usual daily estrogen dosage of 0.1 mg ethinyl estradiol for 2 days followed by 12 days more of the nor-ethisterone alone. She began menstruating at this point and all treatment was discontinued for 3 months. This bleeding indicated that although 5 mg of nor-ethisterone twice daily was sufficient to markedly prolong her usual latent period for withdrawal bleeding, nevertheless it was not sufficient to maintain the endometrium. Accordingly, about 2 weeks later another course of estrogen followed by 10 mg of nor-ethisterone twice daily was given for 14 days and a suction curettement performed. The endometrium showed a marked progestational effect with distinct decidual reaction in many areas.

* We are greatly indebted to Chemical Specialties Co., N. Y., for supplies of 19-nor-ethinyl-testosterone.

† Also called: "nor-ethisterone."

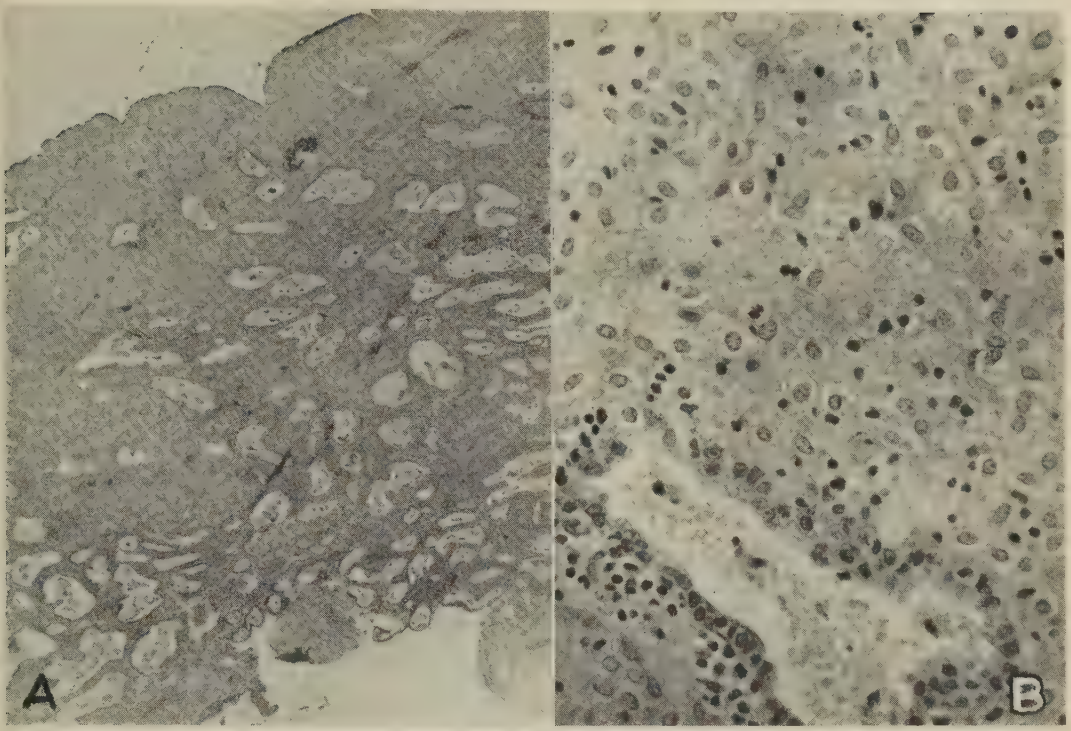

FIG. 1A. Curettage specimen from case 4 after 25 days on 19-nor-ethisterone at 10 mg twice daily.

FIG. 1B. Decidual cells in case 4.

Case 3: M. H. 24-year-old white married female; amenorrhoea of 4 years duration. Menses had been entirely normal (interval 28 days; flow 5 days; napkins 3-4) since 13 years of age. She was given 5 mg of nor-ethisterone twice daily for 5 days and no menses occurred on withdrawal. A second course of 10 mg nor-ethisterone twice daily for 5 days was followed after 3 days by menses lasting about 5 days. Since no spontaneous menstruation occurred during the ensuing 35 days she was given another course of 10 mg of nor-ethisterone twice daily for 5 days and this was followed by menses. Six weeks later she was given ethinyl testosterone at a dose of 40 mg twice daily for 5 days. No bleeding had ensued when the patient was seen 41 days later and further followup was not obtainable.

Although no histological evidence was obtained in this case, 10 mg nor-ethisterone twice daily elicited menses on 2 occasions and half this dose failed to do so. In addition, a 4 times greater dose of ethinyl testosterone also proved ineffective.

Case 4: C.P. 40-year-old white female; carcinoma-in-situ diagnosed by repeated smears and biopsy. She was placed on 10 mg nor-ethisterone twice daily continuously for 25 days while awaiting hysterectomy. No menses intervened during this period. Papanicolaou smears throughout this period remained positive. Quadrant biopsies of the cervix prior to hysterectomy revealed invasive carcinoma of the cervix. Endometrial curettement showed extreme progestational effect with extensive decidual reaction (Fig. 1). Panhysterectomy with node dissection was then carried out.

Conclusions. It is clear from the foregoing observations that 10 mg nor-ethisterone given twice daily represents a reproducibly effective dose in women for the production of marked progestational changes in the endometrium. The data suggest that very probably one half this dose will not be effective. The comparably effective dose of ethinyl tes-

tosterone (ethisterone) would be about 4 times higher(4). Thus the comparative values agree reasonably well in rabbit, monkey, and man(2,3).

1. Tullner, W. W., and Hertz, R., *Endocrinology,* 1953, v52, 359.
2. Hertz, R., Tullner, W. W., and Raffelt, E., *Endocrinology,* 1954, v54, 228.
3. ———, in press.
4. Bickers, W., *J. Clin. Endocrinol.,* 1949, v9, 736.

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Cytological and Weight Changes in Pituitary Gland of the Severely Stressed Rat.* (22282)

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Numerous cytological studies have been made of the anterior pituitary gland following the application of different types of stress. Finerty and Briseno-Castrejon(1) and Brokaw *et al.*(2) reported an increase in the percentage of acidophils as shown by azocarmine after unilateral adrenalectomy in rats. Daughaday, Perry and McBryde(3) associated the acidophils with ACTH secretion in their studies of increased adrenal function in acromegaly. Finerty, Hess and Binhammer (4) reported no increased percentage of acidophils or basophils at intervals of 1, 3, 12 and 24 hours after stress with the azocarmine staining method but did describe an increase in numbers of acid hematein positive cells. No change in the percentage of the basophils by staining with PAS or with aldehyde fuchsin was found. Recently, Knigge(5) reported an increase in percentage of acidophils and argyrophilic basophils 12 hours after severe stress, and concluded that the latter were most probably associated with ACTH production since response to stress occurs in thyroidectomized animals which are characterized by absence of acidophils. Kraus(6) reported a decrease in the number of basophils in the anterior pituitary of Addison's disease patients. Further evidence for the basophils as the cytological source of ACTH was noted by Koneff(7) who found a decrease in the basophils after bilateral adrenalectomy and after ACTH injection in rats. D'Angelo *et al.*(8) noted adrenal hypertrophy during prolonged periods of inanition in the guinea pig. This was accompanied by loss of weight of the pituitary and hypertrophy and hyperplasia of the basophils, increasing with the severity of the inanition. Marshall(9) found ACTH antibody coupled with fluorescent dye to be localized in the basophil cells of the hog pituitary. Recently, Wilson *et al.*(10) reported the characteristic Crooke's hyaline cytoplasmic change of the pituitary basophils associated with hyperfunction of the adrenal cortex, and a progressive loss of acidophils in 100 routine human necropsies.

It was the purpose of the present experiments to reinvestigate histological changes and to study changes in weight of the pituitary gland in order to gain further insight into the effects of a severe stress.

Method. Male rats of the Holtzman strain weighing between 110-130 g were used. They were stressed by immersion (except for the head) in water (containing Aerosol) at 70°C for 5 seconds while under Nembutal anesthesia. All animals were sacrificed by decapitation. The pituitary gland was removed immediately and weighed on a Roller-Smith torsion balance. About half of the glands were then placed in appropriate fixative for future histological study. The rest were placed on previously weighed coverslips, dried in an oven at 30°C for 48 hours and re-

* These studies were aided by contract between Office of Naval Research, Department of the Navy, and University of Texas.

TABLE I. Number of Cells per Field* in Anterior Pituitary after Severe Stress Using Various Staining Methods.

Hr after stress	No. of animals	Acid hematein	Azocarmine			Aldehyde fuchsin		PAS
			Acid.	Baso.	Chrom.	Beta	Delta	
Unstressed	30	21.1 ± .5†	15.7 ± .6	7.0 ± .2	41.9 ± .5	4.6 ± .5	3.6 ± .4	7.8 ± .6
1	35	18.5 ± .5	16.6 ± 1.0	7.7 ± .4	33.5 ± .9	4.2 ± .3	4.4 ± .2	8.0 ± .4
3	32	20.0 ± .8	17.3 ± 1.3	9.8 ± .7	39.3 ± 1.6	3.7 ± .4	5.2 ± .3	8.3 ± .4
12	32	29.4 ± .8	25.5 ± .6	9.9 ± 1.0	51.4 ± 2.5	3.1 ± .4	6.4 ± .5	9.9 ± .5

* Oil immersion 1.25 mm lens and a 20× eyepiece.
† ± stand. error.

weighed. Glands used for histological study were stained by 5 different methods: fixation in Bouin's fluid and stained with hematoxylin for nuclei counts; fixation in Zenker-formol solution and staining with a modified azocarmine stain(11) for cell counts; fixation in formol-sublimate and staining with periodic acid Schiff method for basophils(12); fixation in formol-calcium and staining with acid hematein for phospholipids(13); and fixation in Susa-picric acid and staining with aldehyde fuchsin to distinguish beta and delta cells(14). Cell counts were made on 3 serially cut sections selected at approximately the ¼, ½ and ¾ levels of the gland. The cells were counted in every tenth field for 10 consecutive fields. A standard field was obtained with a 1.25 mm oil immersion Spencer lens and 20x eyepiece. Cell counts were recorded as the number of cells per field.

Results. Exp. I. Ninety-nine pituitaries were prepared for histological study from rats which were sacrificed at intervals of 1, 3 or 12 hours post-stress. Thirty unstressed animals served as controls. Table I shows no significant differences in number of anterior pituitary cells per standardized microscopic field among control, 1 and 3 hour post-stress animals by any of the staining methods. However, 12 hour post-stress animals demonstrated a significant increase in acid hematein positive cells per field. There also were significant changes in the number of acidophils, basophils and chromophobes per standard microscopic field in the 12 hour post-stress animals stained by azocarmine stain as compared to controls. PAS staining of pituitaries of the 12 hour post-stress animals showed an increase in number of basophils per field. The 12 hour post-stress pituitaries showed an increased number of delta cells and a slight decrease in beta cells when stained by Halmi's aldehyde fuchsin method (14). In the 12 hour post-stress groups all cell counts (except beta cells) after various methods of staining show a significant increase in number of cells per field as shown in Tables I and II.

Calculations of percentage of cells stained by the azocarmine method show that there is no significant change in percentage of each type of cell after severe stress. The nuclei counts are similar to the total cell counts after azocarmine staining as shown in Table II. Table I shows total number of basophils after staining by aldehyde fuchsin, azocarmine or PAS to be similar also. The acid hematein stain for acidophils gives consistently higher numbers than for acidophils

TABLE II. Relation of Fresh Pituitary Weight to Total Cells per Field* after Severe Stress.

Hr after stress	No. of animals	Body wt, g	Fresh pituitary wt		Total nuclei/field*	
			Absolute, mg	Relative, mg/100 g	Hematoxylin	Azan
Unstressed	30	119 ± 1.2†	4.11 ± .03	3.44 ± .03	65.6 ± .7	64.7 ± .9
1	35	118 ± 1.2	4.06 ± .02	3.44 ± .02	60.1 ± 1.4	57.4 ± .8
3	32	111 ± 1.6	3.71 ± .03	3.40 ± .02	61.9 ± .9	66.5 ± 1.2
12	32	112 ± 2.0	3.40 ± .03	3.05 ± .02	84.6 ± 2.1	87.2 ± 1.4

* Oil immersion 1.25 mm lens and a 20× eyepiece.
† ± stand. error.

TABLE III. Fresh and Dry Weight of Pituitary after Severe Stress.

Hr after stress	No. of animals	Body wt, g	Rel. wt/100 g body wt		
			Fresh, mg	Dry, mg	Liquid lost in drying, mg
Unstressed	31	121 ± 1.2*	3.41 ± .03	.75 ± .009	2.66 ± .01
1	11	122 ± 1.6	3.46 ± .04	.77 ± .01	2.69 ± .02
12	28	128 ± 2.4	3.01 ± .04	.62 ± .01	2.39 ± .02
Diff. between unstressed and 12 hr post-stress			.40	.13	.27
Relative fluid and solid loss (in %)				32.5	67.5

* ± stand. error.

stained by the azocarmine method as shown in Table I.

General observations of the 12 hour post-stress pituitaries indicated few mitotic figures, and an apparent reduction of cytoplasm in the cells, resulting in an increased nuclear-cytoplasmic ratio.

Results in Table II show no significant change in fresh weight of the pituitary of 1 and 3 hour post-stress animals as compared with controls. There is also no significant change in relative fresh weight per 100 g body weight of 1 and 3 hour post-stress animals. The 12 hour post-stress pituitaries show a significant absolute decrease in weight which is also apparent relative to body weight.

Exp. II. Thirty-nine male rats were stressed and sacrificed at 1 and 12 hours later. Pituitaries were removed, weighed and dried. Determinations of relative weight per 100 g body weight were made of fresh weight, dry weight and weight of fluid lost in drying. The same determinations were made on 31 control animals.

Table III shows no significant changes from controls in the relative fresh weight, relative dry weight and relative weight of fluid lost in the 1 hour post-stress animals. In the 12 hour post-stress animals there was a decrease in relative fresh weight of the pituitary per 100 g body weight similar to that in Exp. I. The relative dry weight and relative weight of fluid lost in drying of the 12 hour post-stress animals show a decrease compared to controls. Table III also shows that 32.5% of the difference in weight between the controls and 12 hour post-stress animals is due to loss in solids and 67.5% of the difference is due to a loss in fluid.

To determine the effects of a severe stress on the weight of an organ of similar anatomical position which has little endocrine function, the brains of controls and 12 hour post-stress animals were removed, weighed, dried and reweighed. No change in fresh or dry weight of the brain was found as a result of severe burns suggesting that the two organs respond differently.

Discussion. Our results confirm previous reports of increase in number of acid hematein-positive cells after stress with no change in percentage of azocarmine staining acidophils(4). This severe burn stress after 12 hours induces some increase in number of all cells per microscopic field, regardless of staining method. This is probably a result of general shrinkage of the pituitary gland, as indicated by weight reduction and increased nuclear-cytoplasmic ratio. Such a finding points out a variable factor which must be considered in interpretation of pituitary cytological studies whenever there is a possibility of pituitary anhydremia.

Measurements of water content of pituitaries after stress indicate that about 68% of the weight reduction at 12 hours post-burn is a result of dehydration. This is compatible with hemoconcentration usually seen in burned patients due to edema and resulting anhydremia. Since the same fluid changes were not found in the brain it is concluded that the pituitary is one of the organs specifically affected by anhydremia, possibly because of its oral epithelial origin.

About 32% of pituitary weight reduction at 12 hours post-burn is a result of loss of solids. Since solids make up only one-fifth of total weight of the gland, it is apparent

that their loss is a significant aspect of the response to stress, amounting to an actual loss of 17% of all solids present. Possible factors responsible for the loss of solids may be loss of diffusible cellular proteins, loss of mineral ions to the blood, or excess secretion of pituitary hormones.

Summary. Effects of severe stress, immersion in water at 70°C for 5 seconds, were studied on the male albino rat in relation to response of the anterior pituitary. Using 5 different staining methods, no significant changes in *percentage* of acidophils, basophils or chromophobes were found 1, 3 or 12 hours after stress, although there were marked increases in *number of cells per field* in the 12 hour post-stress rats. Fresh weight of the pituitary showed no significant change in 1 and 3 hour post-stress animals, but the 12 hour group displayed a marked reduction, which was evident on both an absolute and a relative to body weight basis. Comparison of relative dry weight and amount of fluid lost in drying in unstressed and 12 hour stressed animals revealed that the weight decrease consisted of both fluid loss and reduction in solids.

1. Finerty, J. C., and Briseno-Castrejon, B., *Endocrinol.,* 1949, v44, 293.

2. Brokaw, R., Briseno-Castrejon, B., and Finerty, J. C., *Texas Reports on Biol. and Med.,* 1950, v8, 312.

3. Daughaday, W. H., Perry, W. E., and McBryde, C. M., *J. Clin. Endocrinol.,* 1950, v10, 410.

4. Finerty, J. C., Hess, M., and Binhammer, R., *Anat. Rec.,* 1952, v115, 15.

5. Knigge, K. M., *ibid.,* 1955, v122, 295.

6. Kraus, E. J., *Virchow's Arch.,* 1925, v247, 421.

7. Koneff, A. A., *Endocrinol.,* 1944, v34, 77.

8. D'Angelo, S. A., Gordon, A. L., and Charipper, H. A., *ibid.,* 1948, v48, 399.

9. Marshall, F. M., *J. Exp. Med.,* 1951, v94, 21.

10. Wilson, W. D., and Ezrin, C. F., *Am. J. Path.,* 1954, v30, 891.

11. Briseno-Castrejon, B., and Finerty, J. C., *Stain Technol.,* 1949, v24, 103.

12. Pearse, A. G. E., *Nature,* 1948, v162, 651.

13. Rennels, E. G., *Anat. Rec.,* 1951, v111, 462.

14. Halmi, N. S., *Stain Technol.,* 1952, v27, 61.

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Hemolysis of Human Erythrocytes by a Sulfhydryl Inhibitor, p-Chloromercuribenzoic Acid.* (22283)

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Although sulfhydryl groups have a function in the metabolism and integrity of erythrocytes, its nature is poorly defined. Ingbar (1) reported that cysteine caused sickling of erythrocytes from patients with sickle cell anemia. It was prevented by the addition of heavy metals or p-chloromercuribenzoic acid. Benesch and Benesch(2) have shown that either phenylmercuric hydroxide or mersalyl (salyrgan) caused hemolysis of sheep erythrocytes in a fairly consistent pattern; and that this hemolysis was blocked by sulfhydryl groups but not by sulfur in the disulfide linkage or other forms of sulfur. Dimant, *et al.* (3) more recently have been concerned with the question of *de novo* synthesis of glutathione by erythrocytes. In our previous studies(4) on the feeding of methionine, prothrombin and accelerator globulin decreased in all 3 human subjects. Increased erythrocyte destruction was demonstrated in only one subject.

This present study concerns the relation of certain factors of the red cell environment to hemolysis caused by p-chloromercuribenzoic acid.

Method. The sodium salt of p-chloromer-

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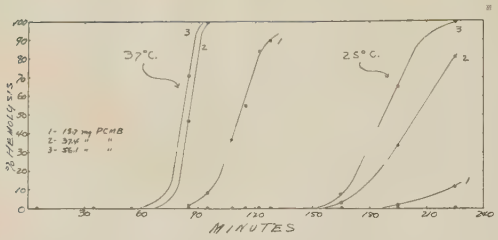

FIG. 1. Effect of concentration and temperature on hemolysis by the sodium salt of p-chloromercuribenzoic acid. Erythrocytes were added at 0 min. There was no hemolysis in the saline control indicated by hemispheres on the base line.

curibenzoic acid† (PCMB) was prepared in 5×10^{-4} molar concentration in 0.9% sodium chloride solution. Erythrocytes were prepared from heparinized blood of normal human donors. The cells were washed 3 times in cool 0.9% sodium chloride solution. After the last washing 0.5 ml of packed cells was added to 25 ml of 0.9% normal saline containing the chemicals studied in the particular experiment. Unless otherwise specified, the cells were incubated at 37°C during the period of observation. The degree of hemolysis was determined by measuring the amount of hemoglobin in the supernatant fluid of a suspension of cells at intervals throughout the study. By relating this figure to 100% hemolysis, obtained by completely hemolyzing a homogeneous suspension of the cells and measuring the optical density in a photometer, the per cent of hemolysis at any time could be calculated. Any existing hemolysis was measured. Correction was made by subtracting this value from the completely hemolyzed sample or the samples obtained at various times. The following equation shows this relationship:

$$\% \text{ hemolysis} = \frac{OD_t - OD_c}{OD_m - OD_c} \times 100.$$

OD_c = Optical density of supernatant immediately after adding red cells.

OD_m = Maximum optical density possible with this concentration of red cells (100% hemolysis).

OD_t = Optical density of supernatant at any time during test.

Zero time was the instant of adding the red cells. The samples of hemoglobin to be measured in the photometer were in a weak solution of ammonium hydroxide. No effort was made to control the pH. During hemolysis the pH of the supernatant fell from 7.6 to 7.3.

† Purchased from Sigma Chemical Co.

Results. 1. *Effect of PCMB concentration*

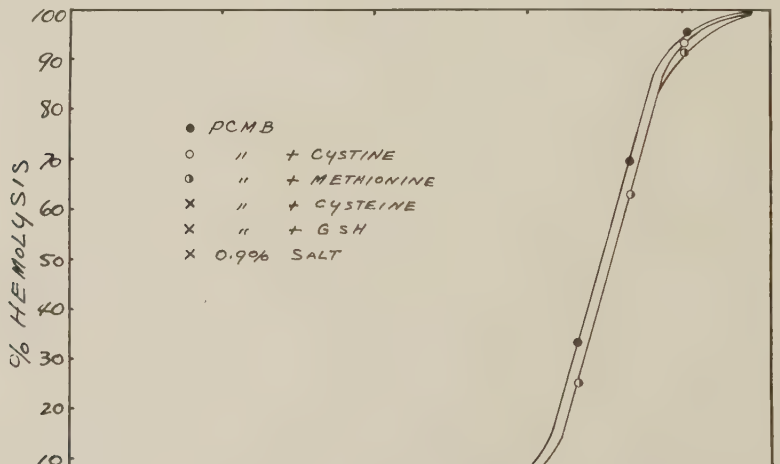

FIG. 2. Amino acids containing sulfhydryl groups inhibit the hemolytic effect of PCMB; those without sulfhydryl are ineffective. There is very little hemolysis of the control erythrocytes in 0.9% saline suspension in 4 hr. Erythrocytes were added at 0 min.

and temperature on hemolysis (*Fig. 1*). Increasing the concentration of PCMB 2 and 3 times the original $5x10^{-4}$ molar resulted in a slight increased rate of hemolysis; and the onset of hemolysis after initial contact with the reagent began at 75 instead of 90 minutes. Decreasing the concentrations of PCMB progressively prolonged the hemolytic process over a period of 3 to 4 hours. Under standard conditions with a concentration of PCMB of $5x10^{-4}$ molar at 37°C hemolysis began in about 90 minutes and was completed in 120 to 130 minutes. This has been a fairly consistent observation with different samples of PCMB and erythrocytes from different donors. Incubation at 25°C lengthened the time before the onset of hemolysis and, once hemolysis had begun, the rate was slower.

II. *Inhibition of hemolysis by amino acids containing –SH groups added to PCMB solution before contact with erythrocytes* (*Fig. 2*). Amino acids containing sulfhydryl groups in equimolar concentrations inhibited the hemolytic effect of PCMB if added to the solution before the erythrocytes. Cystine and methionine were completely ineffective in this inhibition.

III. *Effect of addition of –SH groups after contact of PCMB and erythrocytes* (*Fig. 3*). An equimolar concentration of glutathione was added immediately after the red cells were suspended in the PCMB solution and 30, 60 and 90 minutes later. At one minute and 30 minutes most hemolysis was prevented. There was considerable inhibition of the PCMB effect when glutathione was added as late as 60 minutes. Even at 90 minutes, just before hemolysis was about to begin, the addition of glutathione altered the curve remarkably although hemolysis went to completion.

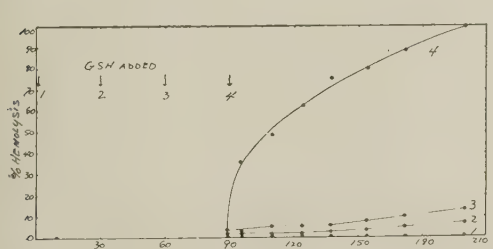

FIG. 3. Inhibition of hemolysis when glutathione added at intervals after contact between erythrocytes and PCMB. Erythrocytes were added at 0 min.

IV. *Effect on hemolysis of washing erythrocytes after contact with PCMB* (*Fig. 4*). Erythrocytes were washed 3 times in cool saline after one minute in contact with PCMB and after 30 and 60 minutes. The washed cells were suspended in 25 ml of fresh saline. Washing the erythrocytes one minute after contact with PCMB solution virtually eliminated the hemolytic effect. Washing after 30 minutes reduced the rate of hemolysis so that there was 20 per cent hemolysis at 210 minutes instead of the usual 90 minutes. Washing at 60 minutes had little influence on the onset and rate of hemolysis except that about 10% of the cells were resistant to hemolysis for over 4 hours. This finding was in contrast to that of the previous experiment when the addition of glutathione at 60 minutes prevented most hemolysis. The supernatant from the initial separation in this study was saved and tested for hemolytic activity by adding another 0.5 ml of red cells. These curves were reciprocals of those in Fig. 4, indicating a progressive loss of hemolytic activity from the solution the longer the erythrocytes had remained in it. The supernatant removed at 60 minutes was free of hemolytic effect. However, contact for 1 minute produced no detectable loss in hemolytic activity. The reaction between erythrocytes and PCMB is not instantaneous but requires a lapse of time.

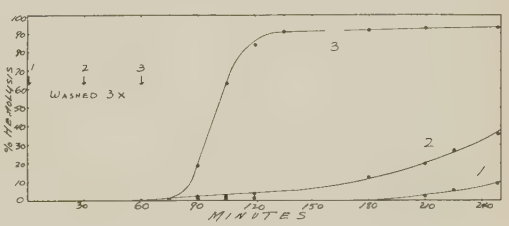

FIG. 4. Incomplete inhibition of hemolysis by washing PCMB from erythrocytes. Red cells were added to PCMB at 0 min.

V. *Exhaustion of PCMB hemolytic effect by exposure of PCMB to single aliquot of red cells* (*Fig. 5*). The usual suspension of red cells in PCMB solution was made and

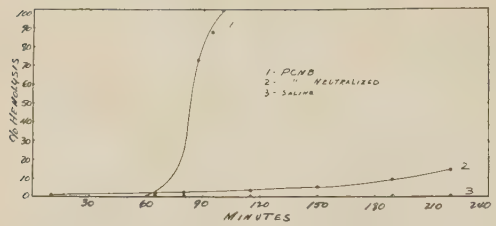

FIG. 5. Exhaustion of hemolytic effect of PCMB by one exposure to erythrocytes. In this chart only, time scale is measured from time when red cells were added to the PCMB in (1) and time when a second portion of red cells was added to the hemolyzed mixture in (2), time when the cell suspension was made in saline in (3).

hemolysis was allowed to proceed. As soon as hemolysis was complete another 0.5 ml of red cells was added to this hemolyzed mixture and more observations were made. There was very little hemolysis of the second portion of red cells, which indicates that the hemolytic effect of PCMB was exhausted by the first aliquot of red cells. This then raised the question as to whether the exhaustion of the hemolytic effect was the result of –SH groups in the cell stroma or inside the cell envelope.

VI. *Exhaustion of PCMB hemolytic effect by hemoglobin but not by stroma* (*Fig. 6*). A suspension of stroma and a hemoglobin solution were prepared by thrice rapidly thawing and freezing 0.5 ml of packed cells. A few milliliters of cold 0.9% sodium chloride solution were added to the hemolyzed mixture which was then centrifuged. The clear supernatant was transferred to a tube and the quantity was made up to 25 ml with 0.9% sodium chloride solution. A small

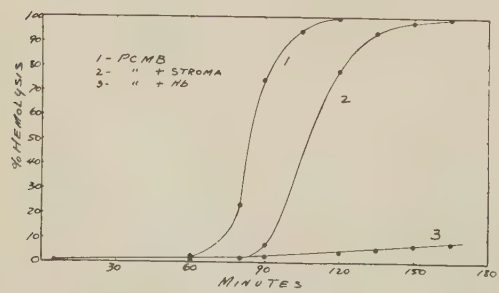

FIG. 6. Inhibition of PCMB hemolytic effect by incubation with hemoglobin solution and failure of inhibition by stroma suspension. Test erythrocytes were added at 0 min.

quantity of fluid containing the stroma was placed in another tube and the volume made up to 25 ml with 0.9% sodium chloride solution. Microscopic examination showed the hemoglobin portion to be virtually free of stroma and the stroma to be concentrated in the other tube. PCMB was added to the two tubes in the concentration of 5×10^{-4} molar followed by incubation for 90 minutes. Then 0.5 ml of erythrocytes was added to each tube and the usual procedure followed. The stroma fraction altered the shape of the curve but only to an insignificant degree. Hemolysis in the other tube was almost completely inhibited by the hemoglobin solution. Since the stroma was not washed the alteration in this curve may have been caused by the small amount of free hemoglobin in the stroma suspension.

VII. *Effect of glutathione plus glucose on the PCMB hemolytic system* (*Fig. 7*). Glu-

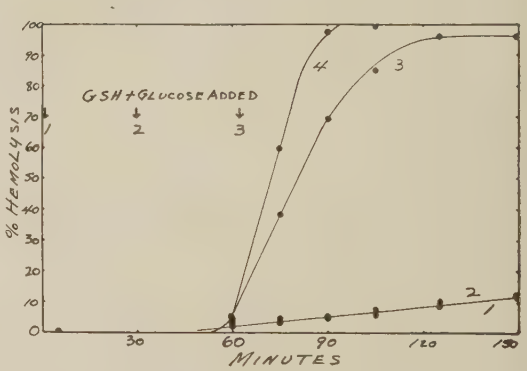

FIG. 7. Failure of glucose to modify inhibition of PCMB hemolysis by glutathione. Erythrocytes combined with PCMB at 0 min. In (4) red cells were suspended in solution of PCMB and NaCl only.

tathione in equimolar concentration with PCMB and glucose to produce a final concentration of 1 mg per 1 ml was added at intervals of 1 minute, 30 minutes and 60 minutes to the red cells suspended in PCMB solution. Glucose had no beneficial effect and was harmful when added at 60 minutes.

Discussion. Organic mercurials which combine with –SH groups cause hemolysis of human erythrocytes. Previous transformation of the organic mercury compound into the mercaptide with cysteine or glutathione

inhibits hemolytic activity. The rate of hemolysis is influenced by temperature(11) as well as concentration of the mercurial reagent. Benesch *et al.*(5) found a striking variation in the reactivity of the –SH groups of crystalline hemoglobin among different species. Those of dogs and man were less reactive than those of sheep. Racker(6) suggested that the difference in rate of reactivity between sulfhydryl groups and reagents may be influenced by properties of adjacent groups on the protein molecule.

That –SH groups are necessary for the integrity of the erythrocyte is a fact. The thiols responsible for this effect are not known nor is the mechanism known. A recent report (5) indicates there are 8 moles of –SH per mole of hemoglobin in man. Previous studies have demonstrated the presence of ergothioneine(7-9) and glutathione(10) within the erythrocyte. The glutathione is in a dynamic state(3) and may traverse the cell envelope. Less is known of the role of ergothioneine.

The necessity of –SH groups for the integrity of the erythrocyte suggests that control of the thiol system may be advantageous in the preservation of whole blood stored for transfusion.

Conclusions. 1. The sulfhydryl reagent, p-chloromercuribenzoic acid, causes hemolysis of human erythrocytes. This action can be prevented by prior reaction with sulfhydryl groups. 2. The hemolytic action of PCMB is reversible by washing cells for as long as 30 minutes after contact with this agent. Reversibility of hemolysis lasts up to 60 minutes after addition of free sulfhydryl group. 3. No protection from PCMB hemolysis was noted with stroma whereas hemoglobin solution was effective. 4. The reaction between PCMB and the –SH group of erythrocytes is relatively slow. 5. A physiologic concentration of glucose offered no protection from hemolysis in this system.

1. Ingbar, S., *Glutathione,* 1954, 228, Academic Press, Inc., New York.

2. Benesch, R. E., and Benesch, R., *Arch. Biochem. and Phys.,* 1954, v48, 38.

3. Dimant, E., Landsberg, E., and London, I. M, *J. Biol. Chem.,* 1955, v213, 769.

4. DeGowin, E. L., Sheets, R. F., Hamilton, H. E., and Carter, J. R., *Trans. Assn. Am. Physicians,* 1955, v68, 118

5. Benesch, R. E., Lardy, H. A., and Benesch, R., *J. Biol. Chem.,* 1955, v216, 663.

6. Racker, E., *Physiol. Rev.,* 1955, v35, 1.

7. Benedict, S. R., Newton, E. B., and Behre, J. A., *J. Biol. Chem.,* 1926, v67, 267.

8. Newton, E. B., Benedict, S. R., and Dakin, H. D., *ibid.,* 1927, v72, 367.

9. Hunter, G., and Eagles, B. A., *ibid.,* 1925, v65, 623.

10. Woodward, G. E., and Fry, E. G., *ibid.,* 1932, v97, 465.

11. Fegler, G., *Nature,* 1952, v170, 624.

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Pyridoxine Responsive Anemia in the Human Adult.* (22284)

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Although pyridoxine is necessary for normal hematopoiesis in dogs(1,2), swine(3-5) and monkeys(6), no evidence is available that abnormal hematopoiesis occurs in humans secondary to a naturally occurring deficiency of pyridoxine or alteration in its metabolism(7,8). In general, the hematologic alterations resulting from pyridoxine deprivation in animals have been characterized by a hypochromic, microcytic anemia with

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high plasma iron; although a moderate leukopenia is usually observed, neutrophilic leukocytosis has also been described. After pyridoxine deprivation abnormal metabolites of tryptophan are excreted following a loading test dose of this amino acid(9,10). The administration of pyridoxine is followed by a well-defined reticulocyte response, rapid fall of plasma iron and restoration of normal leukocyte, erythrocyte and hemoglobin values; concomitantly, the abnormalities of tryptophan metabolism are corrected.

The following observations concern a 35-year-old white male who had a severe hypochromic anemia unresponsive to the usual hematopoietic agents and associated with abnormalities in tryptophan and iron metabolism; these abnormalities were promptly reverted to normal following the intramuscular administration of pyridoxine hydrochloride.

Results. Patient H.S. was first admitted to the hospital in 1947 at 27 years of age, because of weakness and pallor. Except for nocturia, tibial pain and pedal edema that became very bothersome in 1955, the patient's signs and symptoms during entire subsequent course could be directly attributed to decreased hemoglobin level and were abolished by transfusions. No signs or symptoms of glossal, neural, or dermal involvement occurred. His appetite and food intake always remained excellent and he maintained a constant weight. Detailed dietary history showed that the daily caloric intake averaged 2900 and was comprised of 122 g protein, 284 g carbohydrate and 143 g fat; the daily intake of pyridoxine was estimated at approximately 3 mg.† The spleen never became palpable. Admission blood studies showed a hypochromic anemia (4.5 g hemoglobin/100 ml) with normal leukocyte and platelet counts; the leukocyte differential count was within normal limits. Gastric analysis demonstrated 47 units free acid following histamine. A bone marrow biopsy was interpreted as showing some degree of erythrocyte maturation arrest but not consistent with that seen in classic Addisonian pernicious anemia. The anemia was unresponsive to parenteral liver injections (both purified and crude), pteroylglutamic acid, Brewer's yeast, iron and ascorbic acid in the dosages, routes and time duration of treatment indicated in Table I. Transfusions were given as necessary to maintain his hemoglobin at approximately 8 g/100 ml. Continuing studies (roentgenographic studies of the chest, upper gastrointestinal tract, small and large bowel, intravenous pyelograms; basal metabolic rate; stool for ova, parasites, blood excess fat or nitrogen; lymph node biopsy; blood chemistries, electrocardiogram) were done with normal or negative results.

In May, 1948 a spontaneous remission apparently occurred and repeated observations showed red count of over $4x10^6/mm^3$ and hemoglobin of 12 to 13 g/100 ml. He was thereafter asymptomatic and received no therapy until November, 1953 at which time he again noticed onset of weakness and pallor.

Administration of cyanocobalamin and intravenous iron by private physician apparently did not alter progression of symptoms. In December, 1953 the hemoglobin was 8.5 g/100 ml; erythrocyte count $3x10^6/mm^3$, hematocrit, 26%; reticulocyte count, 0.2% and the leukocyte count, $13.2x10^3/mm^3$. The leukocyte differential showed 81% polys, 2% bands, 17% small lymphocytes; the platelet count was $420x10^3/mm^3$; and blood urea nitrogen was 9.9 mg/100 ml. Tests for abnormalities found in various(11) hemolytic states were within normal limits including direct Coombs' test, electrophoresis of hemoglobin, alkaline denaturation for estimation of fetal hemoglobin, Donath-Landsteiner test, cold agglutination test, Ham test, bilirubin partition and total amount: no hemolysins or agglutinins could be demonstrated in the patient's serum in the pH range of 6.5 to 7.5. The indirect Coombs' test was positive at pH 7.5 but consistent with a transfusion subgroup incompatibility. The erythrocyte osmotic fragility was within normal limits; after 24 hours sterile incubation the osmotic fragility had shifted into the range indicating *increased resistance* to osmotic stress com-

† We are indebted to Miss H. Ochi of the Dietetic Service of Crile Veterans' Administration Hospital for these calculations.

TABLE I. Agents Ineffective in Producing a Hematopoietic Response in Patient H.S.

Medication	Route admin.	Amt admin./day	Dates of treatment
Purified liver extract (15 U.S.P. units/ml)	I.M.	1 ml	1/28/47– 2/ 6
Crude liver extract	I.M.	2	4/21 – 4/30
(2 U.S.P. units/ml)	I.M.	5	1/ 8/54– 1/14
Bio-hepulin*	I.M.	2	5/14 – 5/28
Valentine's liquid ex-	P.O.	30	4/21/47– 4/30
tract of liver, U.S.P.	P.O.	90	4/14/54– 4/28
	P.O.	120	4/29 – 5/10
Brewer's yeast tablets	P.O.	20 tablets	2/20/47– 4/30
Ferrous sulfate	P.O.	1 g	2/ 7 – 4/30
Cobaltous chloride	P.O.	180 mg	12/19 –12/24
Pteroylglutamic acid	P.O.	45	3/ 7 – 3/13
	P.O.	100	3/14 – 4/30
	I.M.	15	12/22/53–12/29
	P.O.	30	3/30/54– 4/12
Ascorbic acid	P.O.	150	3/ 6/47– 3/12
	P.O.	200	4/ 4 – 4/30
	P.O.	100	3/30/54– 4/12
Leukovorin	P.O.	30	1/ 2 – 1/ 8
	I.M.	6	12/30/53– 1/ 8/54
Cyanocobalamin	I.M.	100 μg	3/30/54– 4/12
	I.M.	1000	1/ 8/55– 1/30
Pantothenic acid	P.O.	10 mg	12/ 1/47
Cortisone	P.O.	100	1/15/54– 1/20
	P.O.	200	1/21 – 2/ 2
	P.O.	150	1/ 8/55– 1/28
Fresh human plasma	I.V.	1400 ml	6/ 8/54– 6/16
Vit. A	P.O.	75000 units	7/ 8/55– 8/28
D		3000	
Ascorbic acid		450 mg	
Thiamine		15	
Riboflavin		15	
Niacin		750	

* Extract prepared from pregnant mare's liver.

pletely outside the normal range. The erythrocyte mechanical fragilities determined before and after 24 hours of sterile incubation were only slightly increased above normal. An erythrocyte autosurvival time determined by Cr51 demonstrated a half life of 20 days; all the labelled cells had been removed from the circulation by 90 days. These values are significantly below the normal for the method (19). In addition to several agents which had been tried previously, the patient was treated with citrovorum factor, crude liver extract and 1400 ml of fresh plasma intravenously as indicated in Table I. To none of these therapeutic trials did he make any subjective, clinical or hematologic response. His hemoglobin level was maintained at approximately 7 g/100 ml by repeated transfusions of whole blood. In January, 1955 he was readmitted for a trial of cyanocobalamin and cortisone(12): there was no response to this combination of drugs. Transfusions were continued periodically until August, 1955 by which time he had received a total of 113 units of blood. He was then readmitted with an erythrocyte count of $2.86 \times 10^6/mm^3$, 5.9 g hemoglobin/100 ml, hematocrit 22%, leukocyte count $25.5 \times 10^3/mm^3$; the leukocyte differential showed the following percentage distribution: 76 polymorphonuclears, 16 small lymphocytes, 4 monocytes and 4 eosinophils. The mean corpuscular volume was 66 μ^3 and the mean corpuscular hemoglobin concentration, 27%. The erythrocytes on smear showed definitely abnormal variation in size and shape with frequent bizarre forms and

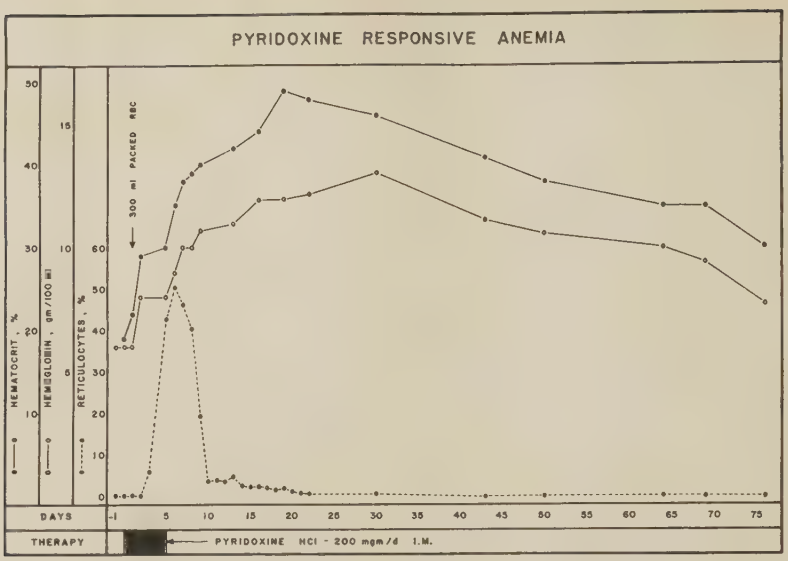

FIG. 1. Initial hematologic response of patient H.S. to intramuscular administration of pyridoxine and subsequent relapse following cessation of therapy.

target cells; the cells appeared hypochromic with decrease in hemoglobin content. During the previous 2 months the patient had been taking one tablet of a vitamin preparation 3 times a day. Each tablet contained: vit. A, 25,000 units; vit. C, 150 mg; vit. D, 1,000 units, thiamine chloride, 5 mg; riboflavin, 5 mg; and niacin, 250 mg. He was also taking quinine, grains 5 p.r.n. usually once every other week for night leg cramps; no other medicines were taken. A transfusion of 300 ml packed red cells was given on the same day pyridoxine hydrochloride, 200 mg intramuscularly daily was started. After 4 doses of this vitamin he noted a subjective improvement in general well-being and on the seventh day after institution of therapy, the reticulocytes had risen to 50.8%. Following this there was a rapid rise in the hematocrit and hemoglobin to maximum values of 49% and 13 g/100 ml respectively, on Sept. 12, 1955. The leukocyte count decreased to 9.2×10^3/mm. As soon as the reticulocytes were found to be elevated significantly, the pyridoxine administration was stopped, 5 doses having been given. By Nov. 14, 1955 the hematocrit had fallen to 27% and the hemoglobin to 7.8 g/100 ml. This response and subsequent relapse are outlined in Fig. 1. Before the response to pyridoxine the patient had complained of very troublesome nocturia, tibial pain and swelling of the feet constant throughout the day. Coincident with the reticulocyte rise the tibial pain disappeared and the edema subsided; shortly thereafter, the nocturia disappeared. After hematologic relapse when the hemoglobin had fallen to 7.9 g/100 ml and the hematocrit to 30%, the patient was started on pyridoxine, 1 mg intramuscularly daily. Except for the 1-tryptophan loading tests as indicated below, no medicines or transfusions were given at this time. No significant reticulocyte response occurred following the 1 mg pyridoxine dose and as indicated in Fig. 2, after 8 days it was increased to 10 mg intramuscularly daily. A reticulocyte peak of 17.7% on the seventh day of this therapy, was followed by a rapid rise in hemoglobin and hematocrit. Again, there had been subjective improvement coincident with the reticulocyte response. On Dec. 14, 1955 the hemoglobin reached 14.1 g/100 ml and the hematocrit 51.9%. The patient received 3 oral loading tests of 4 g 1-tryptophan, the first before pyridoxine therapy was started, the second during the 1 mg dose, and the final one shortly following the reticulocyte response during the 10 mg dose. Measurements of the urinary excretion of kynurenin, kynurenic acid, acetylkynurenin, xanthurenic

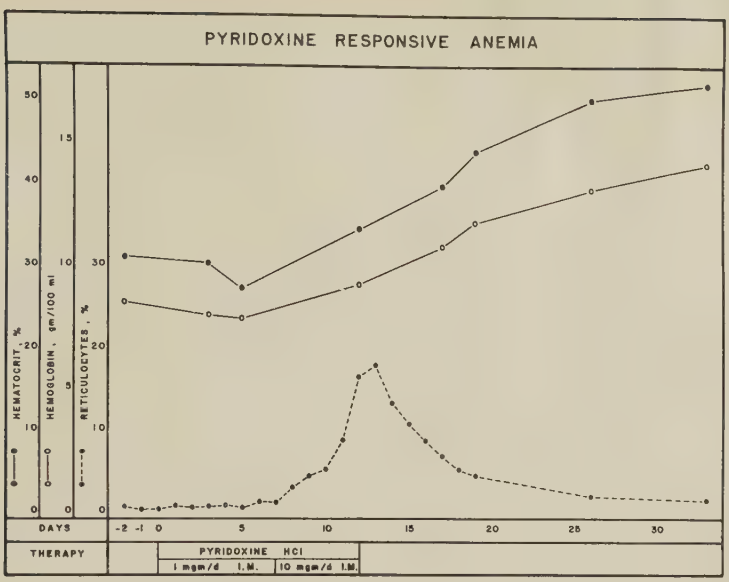

FIG. 2. Lack of hematologic response of patient H.S. to intramuscular administration of 1 mg pyridoxine daily with subsequent response to 10 mg pyridoxine daily. The l-tryptophan loading tests were done on days –2, 5 and 17.

acid, anthranilic acid glycuronide, ortho-aminohippuric acid, and n-methyl-pyridone-5-carboxymid indicated abnormalities of tryptophan metabolism(13). These abnormalities were partly reverted toward normal on the 1 mg pyridoxine dose and completely normalized at the 10 mg level(14). Before therapy the serum iron level was 170 μg/100 ml with a total iron binding capacity of 184 μg/100 ml and an iron binding protein saturation of 92%. Following therapy the serum iron was 67 μg/100 ml; the total iron binding capacity was 214 μg/100 ml and the saturation, 31%.‡

Discussion. Studies concerning pyridoxine have been accomplished in humans by eliminating the vitamin from the diet, counteracting the effect of the vitamin with an antagonist (4-desoxypyridoxine) and administering the vitamin as a therapeutic test(8). Although abnormalities in tryptophan metabolism have been readily produced(10), in only one instance has it been possible to show that pyridoxine deprivation in the human has resulted in altered hematopoiesis manifested by a severe microcytic hypochromic anemia, remediable by pyridoxine alone. Snyderman and associates(15) studied 2 hydrocephalic infants maintained on a synthetic diet devoid of pyridoxine; after 130 days anemia developed in one infant and abnormalities in tryptophan metabolism were demonstrated. These abnormalities disappeared after pyridoxine administration. No instances of naturally occurring abnormalities of hematopoiesis have been reported in humans that were responsive to the administration of pyridoxine. Hunt and associates(16) reported an infant who suffered from intractable convulsions which were controlled by pyridoxine. This infant was also described as having had a moderate anemia during the neonatal period; however, the only values reported for the first 23 days of life are: hemoglobin 13.1 g/100 ml and an erythrocyte count of 4.2×10^6/mm^3.

Employing the metabolic antagonist 4-desoxypyridoxine(17,18) dermatitis, glossitis, neuritis, lymphopenia and alterations in the handling of tryptophan and alanin were

‡ We are indebted to James A. Bowerfind, M.D., of University Hospitals, Cleveland, for these determinations.

produced. However, the anemia that sometimes occurred could not be related to pyridoxine metabolism.

Despite extensive empirical use of pyridoxine as a therapeutic test agent for many different conditions, its essential role in human hematopoiesis has been demonstrated only in the infant maintained on a synthetic diet lacking pyridoxine. The present observations indicate that abnormal hematopoiesis (associated with abnormalities in the metabolism of tryptophan and iron) can occur in the adult human secondary to a naturally occurring alteration in pyridoxine metabolism. Since the dietary intake of pyridoxine was estimated to be normal, the possibilities exist at present that either an absorptive defect or metabolic aberration accounts for the findings in this patient. The nature of the alteration and its role in the steps involved in hematopoiesis are unknown.

Summary. An adult patient is reported with hematologic abnormalities that were unresponsive to the usual hematopoietic agents, and characterized by a hypochromic anemia, leukocytosis, high serum iron and high per cent iron binding protein saturation. Measurements of the abnormal urinary excretion of the metabolites of l-tryptophan following oral loading tests demonstrated an alteration in the metabolism of this amino acid. The clinical and laboratory abnormalities were promptly reverted to normal, and hematological remission followed intramuscular administration of pyridoxine hydrochloride.

1. Fouts, P. J., Helmer, O. M., Lepkovsky, S., and Jukes, T. H., *J. Nutrition,* 1938, v16, 197.

2. McKibbin, J. M., Schaefer, A. E., Frost, D. V., and Elvehjem, C. A., *J. Biol. Chem.,* 1942, v142, 77.

3. Cartwright, G. E., Wintrobe, M. M., and Humphreys, S., *ibid.,* 1944, v153, 171.

4. Cartwright, G. E., *J. Clin. Invest.,* 1944, v23, 926. (Abstract).

5. ———, *J. Biol. Chem.,* 1948, v172, 557.

6. Poppen, K. J., Greenberg, L. D., and Rinehart, J. F., *Blood,* 1952, v7, 436.

7. Wintrobe, M. M., *Clinical Hematology,* Lea and Febiger, Philadelphia, 1952.

8. Vilter, R. W., *J.A.M.A.,* 1955, v159, 1210.

9. Lepkovsky, S., Roboz, E., and Hargen-Smit, A. J., *J. Biol. Chem.,* 1943, v149, 195.

10. Greenberg, L. D., Bohr, D. F., McGrath, H., and Rinehart, J. F., *Arch. Biochem.,* 1949, v21, 237.

11. Harris, J. W., *J. Lab. Clin. Med.,* in press.

12. Arrowsmith, W. R., Burris, M. B., and Segaloff, A., *ibid.,* 1953, v42, 778.

13. Brown, R. R., and Price, J. M., *J. Biol. Chem.,* in press.

14. Harris, J. W., Price, J. M., and Whittington, R. M., to be published.

15. Snyderman, S. E., Holt, L. E., Jr., Carretero, R., and Jacobs, K., *J. Clin. Nutrition,* 1953, v1, 200.

16. Hunt, A. D., Stokes, J., McCrory, W. W., and Stroud, H. H., *Pediatrics,* 1954, v13, 140.

17. Mueller, J. F., and Vilter, R. W., *J. Clin. Invest.,* 1950, v29, 193.

18. Vilter, R. W., Mueller, J. F., Glazer, H. S., Jarrold, T., Abraham, J., Thompson, C., and Hawkins, V. R., *J. Lab. Clin. Med.,* 1953, v43, 335.

19. Ebaugh, F. G., Jr., Emerson, C. P., and Ross, J. F., *J. Clin. Invest.,* 1953, v32, 1260.

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Acceptance of Tumor Homografts by Mice Injected with Antiserum. II. Effect of Time of Injection.* (22285)

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With certain combinations of transplantable tumors and inbred strains of mice, a normally resistant host will accept a tumor homograft if the animal is injected with preparations of mouse tissues prior to tumor grafting(1-4). The altered response of the host may persist for many months after the

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tissue has been injected, but tissue administered *after* the tumor is grafted does not induce acceptance of the homograft(5). *Antiserums* to mouse tissues, produced in rabbits or mice, also will ensure survival of a tumor homograft when injected into normally resistant mice(6-9). The present report is a study of the survival of tumor homografts in mice receiving antiserum at different time intervals, with respect to the time of tumor grafting. It was undertaken to establish a comparison with the results previously found for the effects of varying the time of injection of *tissue* preparations(5). This comparison is of importance for an understanding of the mode of action of both the tissue injections and the antiserums. A preliminary report of the present findings has been presented elsewhere(10).

Materials and methods. The transplantable tumor used as the homograft was Sarcoma I (SaI) which is indigenous to the inbred A strain of mice. Hosts were mice of the C57BL/6Ks strain (a C57Black subline), which is genetically unrelated to the A strain. The tumor grows progressively in 100% of strain A mice, and very rarely in an untreated C57BL/6Ks mouse. (A full description of the properties of this tumor has been given previously(11).) All mice were 2 to 4 months old at the start of the experiments. *Antiserums* were produced in rabbits and C57BL/6Ks mice. The rabbits received injections of either freshly secured spleens of strain A mice or freeze-dried SaI tumor. The tissues were prepared as homogenates in sterile 0.85% saline, in an amount of approximately 20 mg/ml dry weight and the homogenates were centrifuged at 4500 x g. The supernatants thus obtained were used for intravenous injection, and the sediments for intraperitoneal injection. The rabbits received a total of 6 injections, spaced 4 days apart. Except for the first injection, in which supernatant was given intravenously, the sediment was injected in the morning, and the supernatant on the afternoon of the same day, to minimize anaphylaxis. The amounts of sediment injected per rabbit were, respectively, 1.0, 1.5, 2.0, 2.0, and 2.0 ml; all injections of supernatant were in the amount of 0.5 ml. The rabbits were bled from the heart while under nembutal anaesthesia. The serums were separated by centrifugation, freeze-dried and stored *in vacuo,* under refrigeration. For use in the experiments, the serums were reconstituted to the original volume with sterile distilled water. They were then heated at 56°C for 1 hour, since it had been found (12) that unheated rabbit antiserum may induce severe shock in mice, with subsequent death, and that the shock is greatly reduced by heating the serums. At the time that they were reconstituted, the serums had been in storage for 2 years and 8 months. In mice, centrifuged supernatants of freeze-dried or frozen SaI tumor were administered in 5 intraperitoneal injections of 0.5 ml each, over a period of 2½ weeks. Each mouse received a total of approximately 7.5 mg dry weight of tissue. Twelve days after the last injection, the mice were bled by incision of the lateral tail vein. An additional bleeding was made 1 month later, after a single "booster" injec-

TABLE I. Effect of Time of Injection of Rabbit Antiserums on Survival of Homografts of a Strain A Tumor in C57BL/6Ks Male Mice.

Interval between inj. of serums and t g*	No. mice dying with tumors; inj. with antiserums from rabbits No.† K3	208
Serum inj. before t g		
28 days	0/ 5	1/5
7 "	0/ 5	4/5
3 to 6 hr	10/10	5/5
	Serum from rabbits No. 2P31	2P26/304‡
3 to 6 hr before	5/ 6	13/13
Serum inj. after t g		
1 day	2/ 6	3/ 5
2		0/ 5
3	0/ 5	
5		0/ 5
7	0/10	

	Normal rabbit serum	Rabbit antiserum to guinea pig spleen	No inj.
4 hr before t g	0/6	0/6	0/27

* t g = tumor grafting.

† Numerators are No. dying with progressively growing tumors; denominators, total No. of animals in each group.

‡ Pooled serum from 2 rabbits.

tion of an equivalent of approximately 4 mg dry weight of tumor supernatant. All the serums were pooled and stored at −26°C. In some of the experiments, the isoantiserums had been produced shortly before they were used; in others they had been in storage for 10 months. The older serums were fully as effective as the fresher ones. Antiserums were administered intraperitoneally to the test animals. The homografts (SaI) were inoculated subcutaneously by trocar, under aseptic conditions, in the suprascapular region. Growth of the grafts was followed by periodic palpation until the mice either died with a progressively growing tumor, or remained without an evident growth for a consecutive period of 2 months, at which time the animals were classified as "negative."

Results. Serums produced in rabbits. (Table I). Four different lots of antiserums were tested, 3 of these from 3 different rabbits, and the fourth a pooled serum from 2 other rabbits. Rabbit K3 (Table I) had been immunized with the strain A tumor, SaI; the other rabbits were immunized with spleen from strain A mice. The serums from rabbits K3 and 208 were administered in 2 injections, per mouse, of 1 ml each, given 24 hours apart. The other 2 serums were administered in a single injection of 1 ml per mouse. The following points are evident from the data: (a) the best results, in terms of homograft survival, were obtained when the antiserum was injected shortly before tumor grafting; and (b) antiserum injected more than one day after tumor grafting did not effect homograft survival. There is a positive correlation between the relative strength of the antiserums from rabbits 208 and K3 and the length of time that their effectiveness remained in evidence when injected prior to tumor grafting. The serum from rabbit 208 exhibited both a higher complement-fixing titer and hemagglutinating titer (with red cells from strain A/Ks mice) than did the serum from rabbit K3. The data of Table I show that the serum from rabbit 208 exhibited some effect at an interval as long as 28 days between its injection and subsequent tumor grafting, while no homografts survived in the mice that received the K3 serum at as short an interval as 7 days before tumor inoculation. However, that there was some effect of the K3 serum given at this time interval was shown by the markedly larger transient growth of the grafts in this group of mice, and their longer persistence before regression, as compared with uninjected controls.

That the results described are due to specific antibody in the passively transferred antiserum is further shown by the lack of effect of normal rabbit serum, or rabbit antiserum to guinea pig spleen, and by data from a previous study on serum fractions(8).

Serums produced in C57BL/6Ks Mice (Table II). The isoantiserum was adminis-

TABLE II. Effect of Time of Injection of Isoantiserum, produced in C57BL/6Ks Mice, on Survival of Homografts of a Strain A Tumor in C57BL/6Ks Mice.

Interval between inj. of serum and t g*	No. mice dying with tumors ♀	♂
Serum inj. before t g		
9 wk	0/ 5	0/ 5
5	0/ 5	3/ 5
1	5/ 5	5/ 5
4 to 6 hr†	14/15	20/21
Serum inj. after t g		
1 day	—	5/ 5
3	—	5/ 5
7	—	4/ 5
Normal C57BL/6Ks serum, 4 to 6 hr before†	0/ 5	0/17
Nothing inj.	0/ 5	0/10

* t g = tumor grafting.
† Data of 4 experiments.

tered in a single injection of 1 ml per mouse. As with the antiserums produced in rabbits (Table I), the effect of the isoantiserums was more marked as the time interval of their injection, prior to tumor grafting, was decreased. The effectiveness of the isoantiserum, however, remained in evidence for a longer time interval than did the rabbit antiserum. With the isoantiserum, there was a marked transient growth of the grafts (as compared with untreated controls) in the mice injected as long as 9 weeks prior to tumor grafting. This was particularly evident in the male hosts, in whom palpable

grafts were still present 18 days after tumor inoculation, while in the untreated controls the grafts had completely regressed by 13 days.

In the group receiving isoantiserum 5 weeks before tumor grafting, there was a marked transient growth of the tumors in all 5 males. In 3 of the 5 males, the grafts resumed progressive growth after a slight regression, with eventual death of the mice. In the other 2 males, eventually classified as "negative," tumor nodules were palpable for 25 days after grafting. The grafts in the 5 females in this group also showed a large growth before regression, as compared with untreated controls, though they did not reach as large a size as in the males.

The difference between the sexes in the rate of growth of the grafts was also evident in the groups receiving isoantiserum 1 week before, or on the same day as, tumor inoculation. There consistently was a more rapid growth of the grafts in the males. Another difference between the effect of the isoantiserum and the rabbit antiserums was the survival of homografts in the mice receiving isoantiserum as long as 7 days *after* tumor inoculation. No homografts had survived in the mice receiving rabbit antiserum more than 1 day after tumor grafting.

Effect of dosage of antiserum and time of administration. The assumption was tested that dosage of antiserum might account for the differences observed between the effectiveness of rabbit antiserum and isoantiserum administered *after* tumor inoculation. This assumption was based on two premises: that more antibody is needed to insure the survival of a homograft after the immune responses of the host had been initiated; and that more antibody of the type ensuring homograft survival would be found, per unit volume, in isoantiserum than in the rabbit antiserum. Two groups of experiments were done, one with the gamma-globulin fraction of rabbit antiserum, and the other with whole isoantiserum produced in C57BL/6Ks mice. The gamma-globulin was used because it was known that it is active in inducing homograft survival (8). Furthermore, it was felt that the mice would tolerate better larger doses of the gamma-globulin than of whole serum, since it had been found that whole rabbit antiserum, even though heated to 56°C, will induce some degree of shock, and that the gamma-globulin fraction does not produce this reaction(12). Gamma-globulin was prepared by salt fractionation of an antiserum to spleen from strain A/Ks mice. It was then freeze-dried and reconstituted to the desired concentrations, on a dry weight basis, with sterile 0.85% NaCl.

The data for 2 separate experiments with gamma-globulin are given in Table III. In the groups receiving globulin at 4 or 7 days after tumor grafting, there is a positive correlation between the amount of globulin administered and the number of mice dying with progressively growing tumors. A marked growth of the grafts, which was initiated several days after the injection of globulin, was observed in almost all the mice in these same groups, whether or not the grafts were destined to grow progressively. In many of the grafts that eventually grew progressively, to death of the host, this spurt in growth was followed by a progressive diminution in size and then a resumption of growth.

TABLE III. Effect of Dosage and Time of Injection of Rabbit Antiserum Gamma-Globulin on Survival of Homografts of a Strain A Tumor in C57BL/6Ks Male Mice.*

Time γ-globulin inj. after † g†	No. mice dying with tumors at γ-globulin dosages/mouse of			
	10 mg (Exp. 1)	5 mg (Exp.1)	1 mg Exp. 1	1 mg Exp. 2
Same day	—	—	5/5	5/5
1 day	5/5	5/5	5/5	—
3	—	—	—	0/5
4	3/4	2/5	1/5	—
5	—	—	—	0/5
7	2/5	0/5	1/5	—
Nothing inj.			1/5 (Exp. 1)	
3, 5, 7, 9 days after t g‡			0/5 (Exp. 2)	

* Gamma-globulin obtained by fractionation with $(NH_4)_2SO_4$.
† t g = tumor grafting.
‡ 4 inj./mouse; 1 mg/inj.

The experiment with the isoantiserum produced in C57BL/6Ks mice was carried out at 3 different dosage levels of serum, the amount

administered per mouse being given as a single injection of either 1.0 ml, 0.5 ml, or 0.25 ml. The data are presented in Table IV.

TABLE IV. Effect of Dosage and Time of Injection of Isoantiserum, Produced in C57BL/6Ks Mice, on Survival of Homografts of a Strain A Tumor in C57BL/6Ks Male Mice.

Time serum inj. after tumor grafting	No. mice dying with tumors, at serum dosages/mouse of		
	1 ml	0.5 ml	0.25 ml
2 days	5/5	5/5	5/5
4	4/5	3/5	2/5
7	3/5	3/5	1/5
10	1/5	3/5	2/5
14	0/5	0/5	0/5

There is no clear-cut evidence of differences due to dosage. In the groups of mice that died with tumors, and that had received antiserum 4 days, or longer, after tumor inoculation, there characteristically was an initial growth followed by a diminution in the size of the grafts, even for some time after the mice had received the antiserum, followed by a gradual resumption of growth. This experiment demonstrates, incidentally, that the grafts may remain viable in the foreign host for at least as long as 10 days after inoculation, a finding which is in agreement with previously published reports(5,13).

Discussion. The time relationships obtained with the antiserums are in contrast with those obtained in mice injected with tissue extracts(5). Homografts survived in mice inoculated with a tumor graft as long as 11 months after the mice had received an injection of tissue. On the other hand, tissue injected more than one day *after* tumor grafting did not induce homograft survival. With the *antiserums,* there was a diminution of their effectiveness as the interval of time between the administration of the serum and the subsequent grafting of the tumor was increased. This undoubtedly is attributable to the continuous elimination of the serum by the host. The injection of antiserum as long as 4, 7, or 10 days *after* tumor grafting ensured survival of a significant proportion of the homografts.

These observations, together with data obtained with cortisone-treated mice(9,14), indicate that the production of antiserum in mice pretreated with tissues may be a necessary condition for the survival of the tumor homografts. (Though the data show that the effects of the antiserums produced in rabbits and mice are the same, the possibility that there are qualitative differences in their mode of action is not ruled out.)

Three alternative hypotheses may be considered as to the mode of action of the antiserum. (a) The antiserum "coats" the specific tumor antigens which normally would evoke a "homograft reaction" by the host. Such coated antigens presumably would not stimulate the tissues (lymph nodes(13), and possibly other tissues) which otherwise would react to destroy the graft. (b) The antiserum acts directly on the reactive centers (lymph nodes, etc.) of the host to block the "homograft reaction," whether or not the animal had been previously exposed to the immunizing stimulus of a tumor inoculum. (c) The antiserum acts directly on the graft, inducing an adaptive alteration which permits the graft to survive in an otherwise hostile environment.

Our findings make hypothesis (a) untenable, since antiserum injected at 4, 7, or 10 days *after* tumor inoculation, led to survival of the grafts. That these time intervals should be sufficient to develop the immune responses of the host has been shown by Mitchison(13), who demonstrated that immunity to a tumor homograft could be passively transferred by lymph nodes taken from mice 3 to 10 days after the donors had been exposed to the immunizing stimulus of a homograft inoculum.

Whether hypothesis (b) is valid cannot be established from the data of the present paper. However, from data of other experiments it is believed that this hypothesis is open to question. A fuller consideration of this point has been given elsewhere(14).

The possibility that hypothesis (c) is reasonable is supported by the reports of Barrett and Deringer(15,16), and our own data (12), that a tumor homograft that survives in mice of a "foreign" strain loses some of its transplantation specificity, since it will grow

progressively when grafted to untreated mice of inbred strains in which it normally would be rejected. Barrett and Deringer used a transplantable tumor, indigenous to the C3H strain, that had been grown in F_1 (C3H x C) mice. Though the tumor grew in 100% of the F_1 mice, these hosts can be considered as "foreign" to the tumor to the extent that half of the animals' heredity came from strain C parentage. When grafts derived from the "F_1" hosts were implanted into backcross (F_1 x C3H) mice, three times as many mice died with progressively growing tumors, as compared with backcross mice receiving their grafts directly from C3H mice. Barrett and Deringer(15) concluded that " . . . for the moment it is best to describe (the change in the "F_1" tumors) more loosely as an induced adaptation in the tumor." In our own case, the altered grafts came from a strain of mice in which survival of the tumors had been induced by pretreating the hosts with freeze-dried tumor tissue. On subsequent transplantation, the tumors grew progressively in untreated mice of an inbred strain which normally rejects the tumor. However, for reasons unknown, this change in graft specificity was lost after 7 transplant generations. An accelerated tumor growth in mice pretreated with killed tumor tissue, even in animals of the strain to which the tumor is indigenous, has been reported by Casey, Laster, and Ross (17). This characteristic was retained in subsequent transplant generations. Whether these examples are pertinent to our case would depend both upon the demonstration of the production of antiserum under the experimental conditions used by these investigators, and the effects of such an antiserum, if it is produced, upon the growth characteristics of the tumor grafts.

Another point brought out by our data is that the effects of the antiserums injected prior to tumor grafting are indicated not only by those cases where the grafts grew progressively, with eventual death of the host, but also by the numerous instances in which there was a marked transient growth of the grafts before they regressed. The latter cases are most probably an expression of subminimal doses of antiserum present in the host at the time of tumor inoculation. It may be assumed that the number of tumor cells affected by the antiserum was insufficient to ensure continued progressive growth of the grafts.

In conclusion, it is emphasized that a definitive answer as to the mode of action of the antiserum must await further experimentation, and that the mechanisms involved may be concerned with processes as yet unknown, and perhaps other than those that have been considered in this paper.

Summary. Passively transferred antiserum to mouse tissues, produced in rabbits or mice, will induce the survival of a tumor homograft in mice. The best effect, in terms of homograft survival, is obtained if the antiserum is administered to the prospective host shortly before, or shortly after, tumor grafting. The possible mode of action of the antiserum in ensuring homograft survival is considered.

1. Snell, G. D., Cloudman, A. M., and Woodworth, E., *Cancer Res.,* 1948, v8, 429.

2. Kaliss, N., and Snell, G. D., *ibid.,* 1951, v11, 122.

3. Snell, G. D., *J. Nat. Cancer Inst.,* 1952, v13, 719.

4. Day, E. D., Kaliss, N., Aronson, A. I., Bryant, B. F., Friendly, D., Gabrielson, F. C., and Smith, P. M., *ibid.,* 1954, v15, 145.

5. Kaliss, N., and Day, E. D., PROC. SOC. EXP. BIOL. AND MED., 1954, v86, 115.

6. Kaliss, N., and Molomut, N., *Cancer Res.,* 1952, v12, 110.

7. Kaliss, N., Molomut, N., Harriss, J. L., and Gault, S. D., *J. Nat. Cancer Inst.,* 1953, v13, 847.

8. Kaliss, N., and Kandutsch, A. A., PROC. SOC. EXP. BIOL. AND MED., 1956, v91, 118.

9. Kaliss, N., *Annals N. Y. Acad. Sci.,* 1955, v59, 385.

10. Kaliss, N., *Transpl. Bull.,* 1955, v2, 8.

11. Kaliss, N., Borges, P. R. F., and Day, E. D., *Cancer Res.,* 1954, v14, 210.

12. Kaliss, N., unpublished data.

13. Mitchison, N. A., *J. Exp. Med.,* 1955, v102, 157.

14. Kaliss, N., *Annals N. Y. Acad. Sci.,* in press.

15. Barrett, M. K., and Deringer, M. K., *J. Nat. Cancer Inst.,* 1950, v11, 51.

16. ———, *ibid.,* 1952, v12, 1011.

17. Casey, A. E., Laster, W. R., and Ross, G. L., PROC. SOC. EXP. BIOL. AND MED., 1951, v77, 358.

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Failure of Zymosan to Increase Survival in X-Irradiated Mice.* (22286)

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Ross *et al.*(1) stated that the intravenous injection of zymosan in rats and mice was beneficial in radiation injury if given prior to irradiation, and detrimental if given afterwards. Although the effect obtained was related to the dose administered, the dosage range was not given. The mode of action of zymosan is related to its ability to combine with properdin, decreasing then increasing the serum content of this material. Properdin increases survival of irradiated animals by decreasing the post-irradiation bacteremia. Pillemer and Ross(2) gave information concerning the effects produced by doses of zymosan varying from 5 to 125 mg/kg. Finally Pillemer(3) reported that small intravenous doses of zymosan, administered to CF 1 mice, 24 hours pre- or post-irradiation, protected 70% of the animals against an $LD_{100/30}$ day dose of x-irradiation. These investigators did not give the characteristics of the irradiation used or the manner in which the mice were restrained during irradiation. Regardless of the paucity of information, the report of any therapeutic agent which is beneficial in radiation injury requires that further experiments be undertaken by other laboratories. We have conducted such experiments using both low and high doses of zymosan and are unable to substantiate the beneficial effects reported by Ross and Pillemer.

Methods. Six-hundred male, CF 1 mice, weighing an average of 24 g each, were arranged in groups of 20 animals each according to the design given in Table I. The Fleischman yeast zymosan solutions were prepared according to Pillemer and Ross(2). Two series of animals received intravenous injections of 5 and 125 mg/kg respectively, while another series received a 5 mg/kg injection intraperitoneally. Except during irradiation the animals were maintained in an air-conditioned room at 72 ± 5°F and were fed a diet of Rockland pellets supplemented weekly with additional vit. A and D. The 550 r radiation dose was administered from above and below the mice with two 250 KVP Picker Industrial Units operating simultaneously. The technical factors were: 250 KVP; 15 ma; FOD 100 cm; filters, 0.21 mm Cu inherent, 0.5 mm Cu parabolic and 1.0 min Al; HVL 2.02; size of field—total body; r/min measured in air 17.5 to 18.2. Both units were calibrated before and after each experiment with a Victoreen thimble r-meter. The animals were restrained in a plastic cage similar to the one described for guinea pigs (4). The results obtained were analyzed statistically by the Litchfield method(5).

Results. The results obtained with 5 mg/kg and 125 mg/kg of zymosan intravenously are given in Fig. 1 and Table I respectively. Intraperitoneal injection gave results similar to those in Fig. 1. The responses of the saline control groups in all series were similar to results obtained previously with other drugs(6-11). Post-irradiation medication with 5 mg/kg of zymosan either intraperitoneally or intravenously was highly detrimental to the mice, significantly reducing the ST_{50} day and day of total mortality in all cases. Pre-medication did not increase the ST_{50} day and the apparent 20% survival in the 7 day group is of doubtful significance because such effects have been observed in the control groups in past experiments. In fact, only 75% of the control animals of the intraperitoneal series died by the 26th post-irradiation day.

In the series given 125 mg/kg of zymosan, post-irradiation medication on days 1, 3 or 7 was highly detrimental decreasing the ST_{50}

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TABLE I. Effect of 125 mg/kg of Zymosan Intravenously on Survival Time in Irradiated Mice.

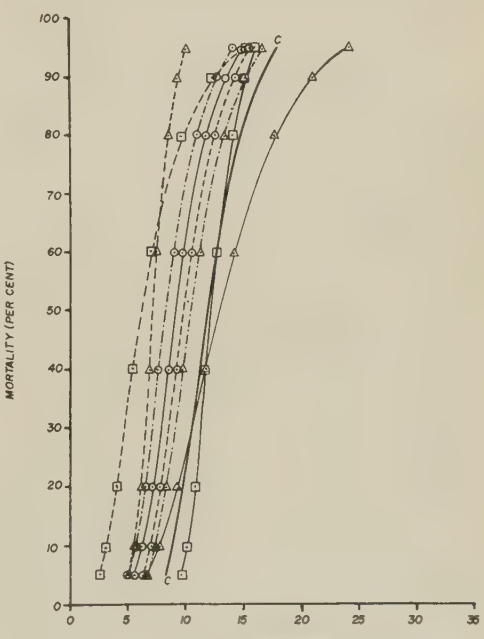
Medication†	ST_{50}* and range in days	Slope and range	Final mortality %	Final mortality Day
Saline control rad day	10.9 (9.45-12.55)	1.39 (1.26-1.54)	90	21
Z, 14 day pre-R	7.55 (6.56- 8.68)	1.39 (1.25-1.54)	95	14
Z, 7 "	10.4 (9.17-11.8)	1.34 (1.22-1.46)	90	21
Z, 3 "	9.65 (7.71-12.1)	1.60 (1.32-1.93)	75	13
Z, 1 hr "	11.8 (5.31-26.21)	1.57 (1.33-1.86)	75	20
Z, " post-R	12.2 (8.19-18.19)	2.31 (1.64-3.26)	65	20
Z, 1 day "	7.35 (6.39- 8.59)	1.38 (1.25-1.53)	100	18
Z, 3 "	5.55 (5.31- 5.85)	1.12 (1.08-1.16)	100	9
Z, 7 "	9.3 (8.65-10.0)	1.18 (1.12-1.25)	100	12
Z, NR control	—	—	0	21

* ST_{50} = Day on which 50% of animals alive. All values at P, 0.05; 20 animals per group.
† Z = Zymosan; rad day = radiation day; pre-R = pre-irradiation; post-R = post-irradiation; NR = non-irradiated.

day as much as 5.5 days over the control value. Pre-irradiation medication on days 14 or 3 also significantly reduced the ST_{50} day. Injections given 7 days or one hour pre-irradiation or one hour post-irradiation produced no significant increase or decrease in the ST_{50} day. However, total survivals in the latter 2 groups were 25 and 35% respectively as contrasted to only 10% survival in the saline control group. The statistical significance of these total survival figures is doubtful because of previous work as well as the 25% survival in the saline controls in the intraperitoneal series. This is also borne out by the wide ranges of both the ST_{50} days and the slope values.

Discussion. There can be little doubt that zymosan, in the doses and modes of administration reported herein, is not beneficial to irradiated mice. Smith(12) has also obtained similar results in mice. Thus, the work of Ross and Pillemer(1-3) cannot be confirmed. However, the conditions under which these investigators conducted their experiments have not been completely reported so that it is not possible to set up an exact duplicate of their work. On the other hand, the statistical design and overall range of conditions of the experiments reported herein should have been sufficient to uncover any beneficial effects which zymosan might have in radiation injury. Insofar as the mechanism of action of zymosan on the properdin system is concerned, the present work actually gives no insight into the problem because properdin titers were not measured. The increased rate of mortality observed with post-irradiation medication might have been the result of depletion of properdin in the blood. However, previous work from this laboratory(6-11) indicates that the irradiated animal reacts adversely to medication during the first post-irradiation week. Compounds like vit. E which are non-toxic in normal animals cause an increase in both the rate of and total mortality, if given after irradiation(7).

Summary. Pre-medication with 5 mg/kg of zymosan intravenously or intraperitoneally did not significantly increase survival in ir-



EFFECT OF ZYMOSAN ON MORTALITY OF IRRADIATED MICE. TREATMENT: SALINE CONTROL——, 5 MG/KG. I.V. ZYMOSAN 14 DAY PRE⊙—⊙, 7 DAY PRE △—△, 3 DAY PRE □—□, 1 HOUR PRE ⊙- - -⊙, 1 HOUR POST △- - -△, 1 DAY POST □- - -□, 3 DAY POST ⊙···⊙, 7 DAY POST IRRADIATION △···△

FIG. 1.

radiated mice. With intravenous injections of 125 mg/kg on pre-irradiation days 14 and 3, a significant reduction in the ST_{50} day was observed. Similar results were obtained with both doses when injections were given after irradiation. The beneficial effects of zymosan in the treatment of radiation injury in mice reported by Ross and Pillemer could not be confirmed.

1. Ross, O. A., Moritz, A. R., Walker, C. J., Wurz, L., Todd, E. W., and Pillemer, L., *Fed. Proc.*, 1955, v14, 418.

2. Pillemer, L., and Ross, O., *Science*, 1955, v121, 732.

3. Pillemer, L., *Trans. N. Y. Acad. Sci.*, 1955, v17, 526.

4. Haley, T. J., and Harris, D. H., *Science*, 1950, v111, 88.

5. Litchfield, J. T., Jr., *J. Pharmacol. Exp. Therap.*, 1949, v97, 399.

6. Haley, T. J., and Rhodes, B., *Science*, 1953, v117, 139.

7. Haley, T. J., McCulloh, E. F., and McCormick, W. G., *ibid.*, 1954, v119, 126.

8. Haley, T. J., McCulloh, E. F., and Heglin, J., *Radiation Research*, 1954, v1, 219.

9. Haley, T. J., McCulloh, E. F., and McCormick, W. G., PROC. SOC. EXP. BIOL. AND MED., 1954, v86, 195.

10. Haley, T. J., McCormick, W. G., and McCulloh, E. F., *ibid.*, 1955, v88, 475.

11. Haley, T. J., McCormick, W. G., McCulloh, E. F., and Heglin, J., *Radiation Research*, 1955, v3, 231.

12. Smith, W., National Institutes of Health, personal communication, October, 1955.

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Effect of Hyaluronidase on the Cat's Brain. (22287)

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The existence of interstitial spaces between elements constituting the nervous system has been assumed. Investigations of Bairati and Mattioli(1,2) confirmed and proved the existence of these interstitial spaces which probably were designed for the flow of cerebrospinal fluid. The experiments of Bairati(3), Bairati and Tripoli(4), Bairati and Patterno (5), Bartoli and Bertaccini(6), and Massari and Marsico(7) demonstrated by histochemical tests on different groups of animals the existence of a ground substance, the main element of which is a mucopolysaccharide or a mucoprotein contained in the interstitial spaces of the nervous tissue. The results of Hotchkiss' reaction were clearly positive in nervous tissue of fishes, amphibia, and lower mammals, whereas the results were doubtful when the test was performed on the brain of birds and higher mammals. These authors studied the behaviour of the nervous tissue under the influence of hyaluronidase and proved that the ground substance is affected by hyaluronidase, which allows a more rapid and extensive diffusion of the injected liquids and an easier mechanical dissociation of fragments of nervous tissue. Thus mucopolysaccharide may be present in interstitial spaces of the nervous substance. Freedman(8) also proved by means of histochemical reactions the existence of a substrate hydrolyzed by hyaluronidase, probably hyaluronic acid, in the nervous system, *i.e.* in the cerebral cortex, nucleus caudatus, and thalamus. Recently, Hess(9) stated too that the ground substance of the nervous tissue is a mucopolysaccharide, rather than a mucoprotein; since it is not hydrolyzed by pepsin, trypsin, or pancreatin. Hess found that hyaluronidase had no effect, and this indicates that this substance is not hyaluronic acid. In the present work, the behaviour of the nervous tissue under action of the hyaluronidase enzyme was examined.

Materials and methods. 13 young adult cats of both sexes were used, with a weight of 1.5-2 kg. All animals were operated on under

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aseptic conditions and ether anesthesia. Symmetrical points of the skull were opened, and after incising the duramater, the following procedures were carried out. In the *first test,* on 2 animals, a superficial wound of the cerebral cortex was made by application of the trephine on both sides of the chosen area for 10 seconds. After stopping the hemorrhage, a square piece of paper 7 mm side length, soaked in physiological saline solution was applied to the control side. On the other side, another piece of paper of the same form and size was applied, which immediately before had been soaked in a recently prepared hyaluronidase solution (30 units of Schering's "Kinaden" in 0.1 ml of physiological serum). Both wounds were covered with a piece of cellophane paper applied to the bone between it and the muscles. After closing the wound, 10 ml of a solution of Merck's trypan blue of 1% in physiological serum was injected intravenously and repeated every 3 hours. The animals were sacrificed 10 hours later by bleeding under anesthesia. In the *second test,* 2 cats were treated in the same way, but the quantity of hyaluronidase used was 10 units. Trypan blue was 20 ml divided into 2 injections separated by 40 minutes. The animals of this group were sacrificed 10 hours later. In the *third test,* cerebral lesions were made by application to the cortex of a square piece of paper, 5 mm side length, soaked with carbol-xylol. This was kept in contact with the cortex for 40 seconds. Thereafter, the symmetrical points of the cortex treated in this way were irrigated with serum at 37° and then the animals were treated exactly as in the first test. One of the animals was sacrificed 2 hours after, and the remaining 2 after a period of 10 hours. In the *fourth test,* 4 cats were treated with hyaluronidase inactivated by heat, the enzyme having been dissolved in serum at 70° and the solution incubated one hour at 70°. The experimental procedure was the same as in the third test. Two animals of this group were sacrificed and the remaining ones 10 hours after. In the *fifth test,* in 2 cats a deep injection of 0.2 ml of trypan blue solution at 1% in physiological serum containing 20 units of Kinaden was injected deeply into the cortex on one side. In a symmetrical point of the cortex of the other side, a 0.2 ml of trypan blue dissolved in physiological serum at 1% was injected, to the same depth and the animals sacrificed after a period of 24 hours. The brains were fixed in an aqueous formol solution at 10% and afterwards examined microscopically with a magnifying glass.

Results. In cats of the first test, a larger diffusion of the dye was seen on the side which had been treated with hyaluronidase (Fig. 1-A). The stain is broader on the treated side than on the control side to which physiological serum had been applied.

In the second test the results were analogous. On the side treated with hyaluronidase the dye extended over a broader area than that which was reached on the control side. The diffusion area was smaller in this test than in the first one.

In the third test, the results were exactly the same as in the previous ones.

In the fourth test, in which the hyaluronidase had been inactivated by heat, the diffusion area of the dye was essentially the same on both sides. (Fig. 1-B).

In the fifth test, the dye formed on the control side a small sediment of quite precise limits while on the side in which hyaluronidase had been injected, it had spread into the adjoining area, in the immediate nervous tissue following the needle's course and forming a halo of pale blue color.

Discussion. The behavior of the nervous tissue in the presence of the hyaluronidase enzyme was also studied by Bairati(10) using the method of injection—at low pressure—of coloring substances (India ink) diluted in solutions of the enzyme with physiological serum. Injections were performed in nervous tissue obtained immediately after death and in living animals. In the technic applied by Bairati, the quantity injected is not exactly determined and the pressure at which the injection is performed cannot be regulated with accuracy, a fact which possibly may influence the degree of diffusion of the coloring substance thus rendering doubtful the interpretation of the results.

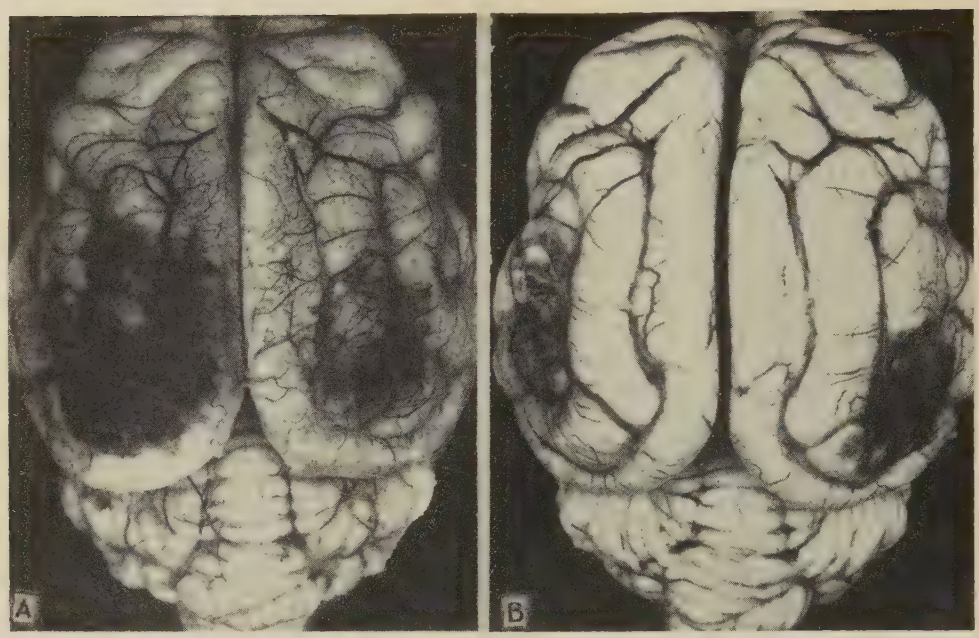

FIG. 1. A. First test. Left side treated with hyaluronidase. Right side (control) treated with physiological serum. Notice broader diffusion of coloring substance on left side. B. Fourth test. Left side treated with hyaluronidase inactivated by heat. Diffusion area of coloring element is approximately the same as on the right (control) side.

The technic used in our experiments avoids any such errors. Passage of the dye takes place through the blood vessels at the same pressure and at the same concentration on both sides, and the animal serves at the same time for test and control.

It can be deduced from our results that in the area of the cortex to which the enzyme was applied, the diffusion of the dyestuff is greater than on the control side. The degree of diffusion was influenced by concentration of the enzyme in the tested solution, and by the time over which it was allowed to act. This effect of the enzyme disappears when it is inactivated.

These results indicate the existence of a substance—probably hyaluronic acid—in the cerebral cortex of the cat which is susceptible to the action of the hyaluronidase.

Summary. 1) The existence of a substance susceptible to the action of hyaluronidase in the cat's brain is demonstrated. 2) The action of hyaluronidase on the cerebral substance is influenced by the concentration of the enzyme and by the duration of its action. 3) When the enzyme is activated by heat, the effects of the diffusion factor on the nervous system disappear.

1. Bairati, A., and Mattioli, G., *Boll. Soc. it. Biol. sper.*, 1951, v27, 127.
2. ———, *Monit. Zool. it. Suppl.*, 1952, v60, 101.
3. Bairati, A., *Att. Acc. Pugliese. Sci.*, 1952, v10, 603.
4. Bairati, A., and Tripoli, G., *Monit. Zool. it. Suppl.*, 1952, v61, 105.
5. Bairati, A., and Patterno, A., *ibid.*, 1952, v61, 107.
6. Bairati, A., and Bertaccini, G., *ibid.*, 1952, v61, 109.
7. Massari, F., and Marsico, G., *ibid.*, 1952, v61, 110.
8. Freedman, B., *Anat. Rec.*, 1953, v115, 265.
9. Hess, A., *J. Comp. Neurol.*, 1953, v98, 69.
10. Bairati, A., *Experimentia*, 1953, v9, 461.

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In vitro Metabolism of DL-Alanine-2-C-14 and Glycine-2-C-14 by *Trichinella spiralis* Larvae.* (22288)

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(Introduced by Donald D. Van Slyke.)
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Recent work from this laboratory(1) has shown *in vivo* incorporation of carbon-14 by immature and encysted *Trichinella spiralis* larvae from mice fed C^{14}-labeled amino acids. When glycine-2-C-14 and dl-alanine-2-C-14-labeled diets were fed to mice with Trichinella infections of 14 and 56 days' duration, the larvae incorporated C^{14} indicating that they were exchanging metabolites with the host. Encysted larvae in 180-day infected mice also incorporated significant levels of C^{14} from the tissues of mice fed glycine-1-C-14 and dl-alanine-1-C-14 diets, showing an active metabolism by well encapsulated muscle larvae.

In the present study, Trichinella larvae were isolated from host tissues by pepsin digest and cultured in media containing dl-alanine-2-C-14 or glycine-2C-14. This report concerns *in vitro* incorporation of C^{14} by muscle larvae and metabolism of C^{14} into larval protein from C^{14}-labeled amino acids.

Materials and methods. Trichinella larvae were obtained from stock mice with infections of 4 months' duration. The muscle larvae were freed from their cysts by pepsin digest, separated from undigested materials and washed to remove soluble digestion products, as previously described(1). The Krebs-Ringer bicarbonate solution was prepared using the procedure outlined by Cohen(2). Normal mouse serum and Krebs-Ringer solution were used to prepare 4 types of media as follows: a) a 50-50 mixture of mouse serum and Krebs-Ringer solution containing glycine-2-C-14; b) a 50-50 mouse serum and Krebs-Ringer solution containing dl-alanine-2-C-14; c) Krebs-Ringer solution with glycine-2-C-14; and d) Krebs-Ringer solution with dl-alanine-2-C-14. A 2 ml sample from each medium was saved to determine C^{14} content prior to incubation with Trichinella larvae. Beckman pH meter determinations of the media gave pH values of 7.68 for the serum Krebs-Ringer media and pH 7.61 for the Krebs-Ringer media. The values for total C^{14} activity and free amino acid concentration in the media are shown in Tables I and II. Aseptic technic was used to prepare 4 series of 50 ml culture flasks containing 10 ml of the C^{14}-labeled media. Approximately 100,000 larvae were introduced into each culture flask by pipette suspension in 0.5 ml of 0.85% saline. Penicillin G Potassium (Lederle) 1,000 units and 1,000 μg of Dihydrostreptomycin Sulfate (Lilly) in 0.2 ml volume were added to each flask to control bacterial growth. Thus, each flask contained a total volume of 10.7 ml of the C^{14}-labeled media. The serum-Krebs-Ringer culture flasks were incubated at 37.5°C for periods of 3, 6, 12, 24, and 48 hours. The Krebs-Ringer culture flasks were incubated at the same time for periods of 6, 24, and 48 hours. During incubation the flasks were shaken at 50 cycles per minute. At the end of each incubation period, the larvae were separated from the culture media by centrifugation at 1,000 rpm. The larvae were washed 6 times in 50 ml distilled water and the supernatant drawn off by aspiration. Live Trichinella larvae settle out rapidly, while the dead larvae, if present, float and are lost in the supernatant. Microscopic examination of the washed larvae just prior to lyophilization revealed only motile larvae. The following procedure was devised to obtain total soluble and insoluble larval protein for C^{14} analysis. Lyophilized larvae (7 mg) samples were weighed in specially modified 12 ml centrifuge tubes. The tubes were fitted with removable base tips. The larval material in the base tip was suspended in 0.15 ml of 10% sodium tungstate

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TABLE I. Influence of Incubation Time upon Glycine-2-C-14 Metabolism by Trichinella Larvae.

Composition of glycine-2-C-14 media	Serum and Krebs-Ringer solution					Krebs-Ringer solution		
Total activity/flask (cpm)	3.808×10^6					3.1576×10^6		
Total wt of free glycine/flask (μg)	(86.7† + 95‡) = 181.7					71.9†		
Hr of incubation	3	6	12	24	48	6	24	48
Total wt in mg lyophilized larvae	40.14	34.11	37.22	31.24	26.09	35.01	36.22	27.80
Total wt in mg larval carbon	15.99	13.92	14.42	12.80	10.71	15.44	14.21	11.16
cpm/mg carbon lyophilized larvae	1,938	3,284	8,145	27,895	47,846	1,481	30,697	58,158
Total activity (cpm) lyophilized larvae*	30,998	45,713	117,451	357,056	512,430	22,867	436,204	649,043

* These values represent total C^{14} content in larvae recovered from each culture flask.
† Added glycine is calculated from No. of counts per flask and specific activity of glycine-2-C-14 (19.965 μc/mg).
‡ Estimated from content of free glycine of normal mouse plasma(5).

TABLE II. Effect of Incubation Time upon Dl-Alanine-2-C-14 Metabolism by Trichinella Larvae.

Composition of dl-alanine-2-C-14 media	Serum and Krebs-Ringer solution					Krebs-Ringer solution		
Total activity/flask (cpm)	1.143×10^6					0.776×10^6		
Total wt of free alanine/flask (μg)	(127† + 285‡) = 412					87†		
Hr of incubation	3	6	12	24	48	6	24	48
Total wt in mg lyophilized larvae	36.26	36.07	36.78	29.74	40.46	33.17	27.89	27.41
Total wt in mg larval carbon	15.85	15.38	15.85	12.77	16.92	14.79	12.26	11.42
cpm/mg carbon lyophilized larvae	190	525	734	901	1,602	214	2,055	2,300
Total activity (cpm) lyophilized larvae*	3,011	8,075	11,633	11,505	27,106	3,165	25,194	26,266

* These values represent total C^{14} content in larvae recovered from each culture flask.
† Added dl-alanine is calculated from No. of counts per flask and specific activity of dl-alanine-2-C-14 (4.08 μc/mg).
‡ Estimated from content of free l-alanine of normal mouse plasma(5).

and precipitated with 1 ml of ⅔ *N* H_2SO_4. The precipitated larval protein was centrifuged into the tip of the tube at 2,200 rpm. The material in the tip was resuspended in 0.15 ml sodium tungstate, precipitated again with acid and centrifuged. The process was repeated once more and the samples dried in a high vacuum oven at 30°C. The centrifuge tips containing the larval protein† were removed and placed in Van Slyke carbon combustion tubes for analysis. All lyophilized larvae and larval protein samples were analyzed for C^{14} using the methods of Van Slyke *et al.*(3,4).

Results. The first experiment was designed to test *in vitro* the ability of *T. spiralis* larvae to metabolize glycine-2-C-14 when cultured in a serum-Krebs-Ringer medium and a chemically defined Krebs-Ringer medium. The total activity (cpm) of C^{14} per flask and period of incubation for each culture are shown in Table I. It is evident from the data that the larvae actively incorporate glycine-2-C-14 throughout the 48-hour period of incubation in both types of media. The larvae cultured in the Krebs-Ringer medium incorporated more C^{14} than the larvae cultured in the serum-Krebs-Ringer medium. This is of interest in that the total activity (cpm) per flask was higher in the serum-Krebs-Ringer medium than in the Krebs-Ringer medium. This may indicate an acceleration of glycine-2-C-14 metabolism when it is the only amino acid present in the medium and also suggests that the larvae utilized unknown metabolites present in the serum. The amounts of serum free amino acid (glycine) and C^{14}-labeled glycine per flask are shown in Table I. When isotope dilution of the C^{14}-labeled amino acid by the free amino acids of serum is considered, the actual uptake of glycine by the larvae in the serum media may be greater than indicated by the C^{14} values. The incorporation of C^{14} by larvae in the Krebs-Ringer culture during the first 6 hours was less than half the amount found in the larvae incubated for 6 hours in the serum-Krebs-Ringer medium. Apparently, the larvae in the chemically defined Krebs-Ringer medium required a period of adaptation to the medium and in the following 6- to 24-hour period of incubation incorporated glycine-2-C-14 at a more rapid rate.

In the second *in vitro* experiment, the metabolism of dl-alanine-2-C-14 by Trichinella larvae was studied. The total activity (cpm) of C^{14} per flask and period of incubation for each culture are shown in Table II. The larvae were taken from the same pool of isolated muscle larvae used in the first experiment. The cultures were incubated at the same time as the cultures in the first experiment. The data in Table II indicate that the larvae were able to metabolize dl-alanine-2-C-14 although not as extensively as found with glycine-2-C-14. During the first 6 hours of incubation, the larvae incorporated more C^{14} from the dl-alanine-2-C-14 serum-Krebs-Ringer medium than from the Krebs-Ringer medium. Although the C^{14} activity (cpm) of the serum-Krebs-Ringer medium was higher than the Krebs-Ringer medium, the 6- and 24-hour period of incubation demonstrated a more rapid rate of C^{14} uptake by the larvae in the Krebs-Ringer culture, than in the serum-Krebs-Ringer culture. If, in the 48-hour serum cultures one assumes that the same proportion of the l-alanine, added with the free amino acids of the serum, is incorporated as of the l-alanine in the added C-14-dl-alanine, the total amount of alanine incorporated is estimated to be about 5 times the amount incorporated in the 48-hour Krebs-Ringer cultures.

Table III shows the per cent incorporation of C^{14} into total larval protein by Trichinella larvae cultured 48 hours in the 4 types of media. A higher concentration of C^{14} was found in the total protein obtained from larvae cultured in either glycine-2-C-14 or alanine-2-C-14-labeled Krebs-Ringer media as compared with the C^{14}-labeled serum-Krebs-Ringer media. Although the total C^{14} level of the serum-Krebs-Ringer media was higher than the Krebs-Ringer media (Tables I and III), the larvae incorporated less C^{14} into protein when they were incubated in the

† The material precipitated by tungstic acid will be referred to as "protein"; it presumably includes lipids, purines and primidines of nucleic acids.

TABLE III. Influence of Composition of Media upon Protein Metabolism of 48 Hour Incubated Trichinella Larvae.

Amino acid	Glycine-2-C-14		Dl-alanine-2-C-14	
Media	Serum and Krebs-Ringer solution	Krebs-Ringer solution	Serum and Krebs-Ringer solution	Krebs-Ringer solution
cpm/mg of lyophilized larvae	19,634	23,362	670	959
cpm in protein from 1 mg lyophilized larvae	13,660	19,712	212	545
% C^{14} incorporated into larval protein	69.6	84.4	31.7	56.8

serum media, indicating that they utilized metabolites present in normal mouse serum.

The C^{14} activity from glycine-2-C-14 was incorporated into larval protein material in higher quantity than alanine-2-C-14 regardless of the media used. Glycine, in addition to its incorporation into protein peptides as glycine, may be converted into other amino acids which are eventually incorporated into protein. Furthermore, there is the possibility of incorporation of glycine into purines and pyrimidines of the nucleo-proteins. The higher values of C^{14} activity from alanine-2-C-14 in nonprotein larval material suggests that the larvae are able to convert alanine into nonprotein components, such a glycogen, glucose-1-PO_4 and others *via* the pyruvate pathway.

Summary. 1. *Trichinella spiralis* larvae incorporated C^{14} when cultured *in vitro* in a serum-Krebs-Ringer medium or Krebs-Ringer medium containing either dl-alanine-2-C-14 or glycine-2-C-14. 2. Carbon-14 analysis of the larvae revealed a progressive uptake of the C^{14}-labeled amino acids in both types of media through 48 hours of incubation. 3. The larvae incorporated more C^{14} activity (cpm) from glycine-2-C-14-labeled media than from dl-alanine-2-C-14-labeled media. 4. Of the total C^{14} incorporated by larvae cultured 48 hours in glycine-2-C-14-labeled media about 70 to 84% was precipitable by tungstic acid, and was presumably chiefly in the proteins. Larvae cultured in the dl-alanine-2-C-14-labeled media incorporated about 32-57% of their total C^{14} content into material precipitable by tungstic acid.

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1. Stoner, R. D., and Hankes, L. V., *Exp. Parasitol.,* 1955, v4, 435.

2. Cohen, P. P., *Manometric Techniques,* Burgess Pub. Co., 1945, 193.

3. Van Slyke, D. D., Steele, R., and Plazin, J., *J. Biol. Chem.,* 1951, v192, 769.

4. Sinex, F. M., Plazin, J., Clareus, D., Bernstein, W., Van Slyke, D. D., and Chase, R., *ibid.,* 1955, v213, 673.

5. Albritton, E. C., *Standard Values in Blood,* Air Force Technical Report, No. 6039, 1951, 99.

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Effect of Intravenous Administration of Lactate on Blood Pyruvate Level in Man. (22289)

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The role of circulating lactate in body metabolism is not entirely clear. On the one hand, the concept of the Cori cycle implies that most or all circulating lactate becomes glycogen in the liver; on the other hand, there is ample evidence that lactate can serve as a source of energy in some organs(1). Knowledge of whether the blood pyruvate

level changes after intravenous infusion of lactate should afford some evidence concerning whether or not lactate enters the general metabolic pool. Accordingly, the former was studied in normal human subjects.

Material and methods. Eight subjects, ranging in age from 18 to 30 years, were used; 3 were women. All were well nourished and had taken, in addition to their usual diets, added amounts of carbohydrate for at least three days before receiving the lactate infusion. The studies were made with subjects recumbent after a fast of 14 hours and a rest of at least half an hour. Each subject was given an intravenous infusion of 17.4 g of sodium d-lactate, dissolved in 500 ml of isotonic sodium chloride solution, in 30 minutes. Blood samples were taken before, and at 15-minute intervals for one hour after, the start of infusion. The method of Barker and Summerson(2) was used to measure blood lactic-acid concentration; the method of Seligson and Shapiro(3) was used to measure blood pyruvic and α-ketoglutaric acid concentrations. In addition, 4 subjects, including one who had received a lactate infusion, were given 50 g of glucose intravenously under similar experimental conditions; blood pyruvate concentrations were measured before and immediately after infusion.

Observations. The blood pyruvate level rose during and after infusion of sodium d-lactate (Fig. 1); the average rise at the end of infusion was 0.55 mg per 100 ml of blood, or 89% of average control value of 0.62 mg per 100 ml. After infusion blood pyruvate level usually fell more slowly than did blood lactate concentration (Fig. 1).

Blood α-ketoglutaric-acid concentration rose in 6 experiments (including 2 in which a slight initial fall occurred). It was unchanged in 1 and fell in 1 during and after lactate infusion (Fig. 2).

Intravenous administration of 50 g of glucose in 30 minutes caused no rise in blood pyruvate level in any of four experiments.

Discussion. Increasing circulating blood lactate concentration in normal man caused a striking increase in blood pyruvate level. There was no good correlation between rise

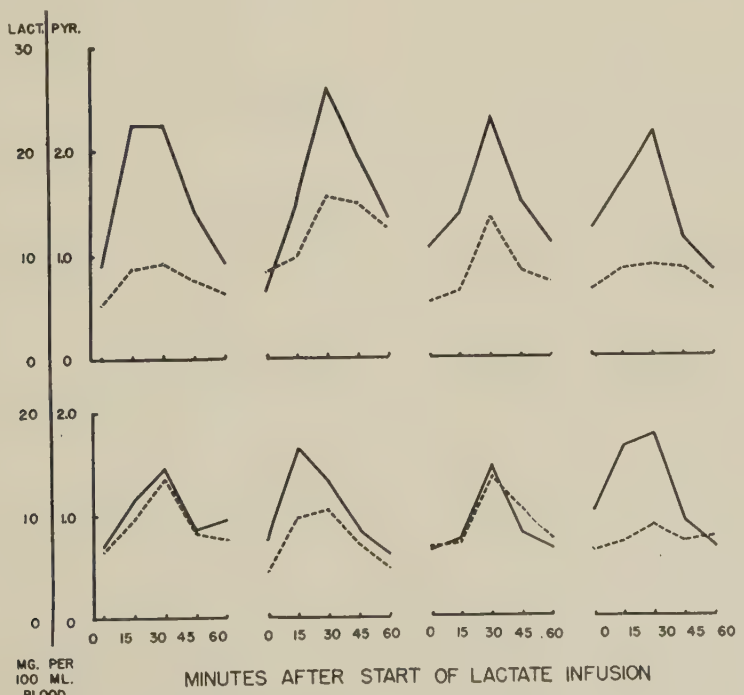

FIG. 1. Changes in blood lactic (solid line) and pyruvic (broken line) acid concentrations during and after infusion of sodium d-lactate.

in blood lactic acid level and that of blood pyruvic acid concentration. Of 3 subjects who showed smallest increases in blood pyruvate concentration, 1 excreted large amounts of lactate in the urine. The other 2, on the other hand, showed large increases in blood lactate; hence urinary excretion of that substance could not account for the small rises in blood pyruvate concentration.

The present data allow no conclusions about quantitative relations between lactate administration and pyruvate formation, for several reasons: 1) Varying amounts of lactate were excreted in the urine; 2) there is no evidence that rate of removal of pyruvate from circulation was the same in all cases; and 3) it is probable that rate of transformation of lactate to pyruvate was not uniform among the subjects. No information is available concerning the site where lactate was changed to pyruvate under conditions of the experiment.

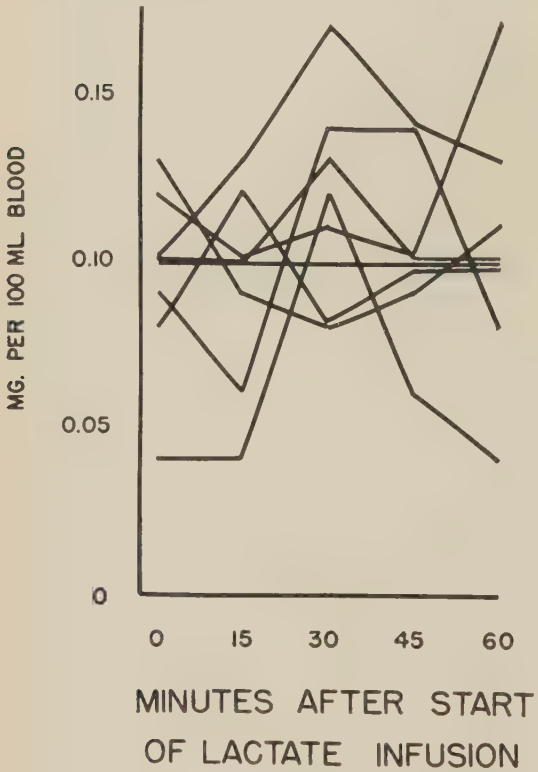

FIG. 2. Changes in blood α-ketoglutaric acid concentration during and after infusion of sodium d-lactate.

It is evident that circulating lactate can contribute rapidly and in significant degree to the general metabolic pool in resting, fasting, well-nourished man. The rise in blood α-ketoglutaric-acid level that usually accompanied the rise in pyruvic-acid concentration supports this conclusion. Actually, the rise in blood pyruvate concentration that occurred after a half-hour infusion of 17.4 g of lactate was far greater than that which occurred after a half-hour infusion of 50 g of glucose. This fact suggests that lactate enters the metabolic pool far more rapidly than does glucose.

Conclusion. The striking rise in pyruvate concentration that accompanies the infusion of lactate in fasting, resting human subjects indicates that circulating lactate contributes to the general metabolic pool.

1. White, A., Handler, P., Smith, E. L., and Stetten, De W., Jr., *Principles of Biochemistry,* McGraw-Hill Book Co., Inc., 1954.

2. Barker, S. B., and Summerson, W. H., *J. Biol. Chem.,* 1941, v138, 535.

3. Seligson, D., and Shapiro, B., *Analyt. Chem.,* 1952, v24, 754.

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Cytological Response in Embryonated Eggs to Inoculums from Cases of Common Cold Infection.* (22290)

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Studies on common cold infections have involved principally transmission studies in human volunteers. Frequently, the results of such studies have been difficult to evaluate because of the variable human susceptibility and the indecisive clinical criteria for diagnosis of the common cold syndrome. Human inoculation experiments have served to demonstrate that common cold infection is a transient air borne viral disease(1,2), limited in pathogenicity to man and ape,(3,4). Propagation of the etiological agent in cultures of human tissues has been reported(5). Propagation of common cold agents in embryonated eggs has been affirmed(6-10) and denied (4,11) but in all instances the decisions rested on the response of human volunteers to experimental inoculations.

Recent observations have indicated that the propagation of influenza and mumps viruses in embryonated eggs was accompanied by an increase in numbers of histiocytes in the allantoic fluid (AF). Parallel studies by hemagglutination and histiocyte counts indicated that both of these effects occurred simultaneously. This non-specific cellular reaction was inhibited by antiserums mixed with the viral agents prior to inoculation, thereby affording some basis for identification of the agent. Using this as a reference point, less well recognized pathogens were studied for similar effect in AF. By following the cytological response in serially passaged AF in inoculated eggs, this effect was demonstrated with serum inoculums from acute cases of clinically diagnosed infectious and serum hepatitis(12,13). Nasal washings from clinically diagnosed cases of common cold were similarly studied in embryonated eggs and increased numbers of histiocytes in the AF were associated with such inoculums(14). Control inoculums from cases of allergic rhinitis have been negative. The experimental data on which these observations were based are described below.

Methods. Standard strains of influenza A, A[1], and B viruses† were each inoculated into the AF of six 9 day embryonated eggs. After 2 days of additional incubation the eggs were stored at 4°C overnight and blood-free AF was aspirated by syringe and needle. A 20 ml aliquot of each pooled AF was centrifuged at 2000 rpm for 20 minutes, the supernatant fluid was quickly decanted, and the sediment was resuspended in the residual fluid. A standard "Breed" loopful of the sediment was transferred to a 1 sq cm area on a clean glass slide. Duplicate smears of each specimen were air dried, stained with Wright's stain, and air dried. The total number of histiocytes in 100 consecutive fields was determined with a 5 X ocular and an oil immersion lens. The histiocytes referred to are mononuclear basophilic cells in which a distinct cytoplasm can be detected. In contrast to the rigid cell wall of the erythrocyte line of cell, the histiocyte was undulant. Blood in the AF was minimized by killing the embryos with refrigeration. Groups of 7 day embryonated eggs were inoculated into the chorioallantoic cavity with allantoic fluid containing mumps virus (Enders strain). Four days later the inoculated eggs were stored overnight in the refrigerator and the AF, collected on the morning, was processed for histiocyte content and hemagglutination titer. Controls for such inoculations consisted of normal AF, fluids from eggs previously inoculated with normal AF, and heated influenza and mumps viruses (61°C/30 minutes). The AF specimens were simultane-

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† Obtained from American Type Culture Collection, Washington, D.C.

ously examined for hemagglutinins with chicken erythrocytes(15). Antiserums from chickens, specifically immunized against the 3 influenza types, were mixed equal parts with viral inoculums and their *in ovo* effects on hemagglutinin production and histiocyte counts were determined. Nasal washings were collected from 21 human patients (medical students) having acute afebrile rhinitis, a syndrome coinciding with the conventional description and history of common cold. These cases occurred during the winter season and by careful selection those having a history of allergic rhinitis were excluded. Nasal washings were collected within 12 hours after onset of illness and a second specimen was collected 2 weeks later when the patient was asymptomatic. Five ml of sterile rabbit serum (2%) in physiological saline was instilled into the nostrils of the reclining patient and the fluid was permitted to run from the nostrils into a petri dish when the patient sat up. The nasal washings were treated with penicillin (1200 units per ml) and streptomycin (100 μg per ml), then 0.1 ml was inoculated into the allantoic cavity of six 7-day embryonated eggs. The shell holes were sealed and the eggs were incubated for 4 additional days at 38°C. The live embryos were then killed by overnight storage at 4°C. The allantoic fluid was collected and examined for histiocytes by the technic described above and the number of histiocytes per 100 oil immersion fields was recorded. Serial passages of AF were made with 0.1 ml into the allantoic cavity of 7-day eggs. Control inoculations consisted of normal allantoic fluid, heated inoculums (61°C/30 minutes), and convalescent nasal washings. Additional control inoculums consisted of nasal washings from 23 individuals with acute allergic rhinitis. The diagnosis was based on a history of allergic episodes following exposure to known allergens and on the microscopic detection of numerous eosinophilic leukocytes‡ in the nasal effluent. The nasal washings were collected in physiological saline and stored at −20°C and subsequent manipulation in eggs followed the technic described above.

It is essential to the accuracy of the test procedure that eggs of superior quality be incubated under carefully controlled conditions, especially with regard to temperature and humidity. Abnormal number of histiocytes were encountered in the AF of uninoculated eggs when they were exposed to high temperatures during the shipment to the laboratory. The eggs used during the cool time of the year were uniformly satisfactory. Experimental procedures were performed at least in duplicate and all fluids were cultured for bacterial content in thioglycollate medium.

Results. The propagation of influenza and of mumps viruses was accompanied by the appearance of hemagglutinins and of histiocytes in the AF (Tables I and II). The cell counts significantly exceeded the normal levels (Fig. 1). Both histiocyte and hemagglutinin production were specifically reduced by treatment of inoculums with homologous antiserums (Table I). Histiocyte counts and hemagglutinins were negative at the same titration end-point in eggs (Table II).

Antibiotic-treated nasal washings from 15 of 21 common cold patients induced increased numbers of histiocytes in the AF of inoculated eggs. Hemagglutinins could not be detected. The embryos did not die as a result of such inoculations. The counts ranged from borderline with 5 specimens to marked increases with 10 specimens (Fig. 1). Subsequent inquiries of the donors revealed that 3 of the negative specimens were collected later than 24 hours after onset of illness. The normal limit of 15 cells per 100 fields was not exceeded with AF from passage of "normal" AF and with AF from uninoculated eggs. The

TABLE I. Serum Neutralization of Influenza Viruses. Detection by histiocyte count and by hemagglutination technics.

Virus	Without serum	+ antiserum for influenza A	A′	B
A	360/ 640*	0/ 0	177/320	140/320
A′	104/2560	224/ 640	1/ 0	143/640
B	192/ 160	312/1280	316/640	0/ 0

* No. of histiocytes per 100 fields/reciprocal of hemagglutination titer.

‡ Stained with "Hansel" stain, Lide Laboratories, St. Louis, Mo.

TABLE II. Titration of Inoculums in Embryonated Eggs.

Agent	No. of histiocytes per 100 oil immersion fields							
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}
Influenza A	—	200+/1280*	180/2560	266/2560	200/2560	0/ 0	—	—
" A′	—	200+/2560	237/2560	210/2560	288/2560	152/1280	102/320	0/0
" B	—	182 /1280	151/1260	154/ 640	3/ 0	—		
Mumps	46/160	47 / 160	3/ 0	12/ 0	—			

* Histiocyte count/hemagglutinin titer.

effect of nasal washings from the 11 convalescent patients who were available contrasted markedly with that of their specimens collected during the acute stage of illness (Fig. 2): none of the convalescent specimens engendered counts in excess of the normal limit. These were collected and processed by the same method as the acute specimens. Simultaneous inoculations into groups of eggs of untreated and of heated nasal washings indicated that the agent responsible for the high histiocyte count was thermolabile (Fig. 3).

Smears from allergic rhinitis tissues revealed numerous eosinophilic leukocytes, whereas smears from the common cold cases contained numerous epithelioid mononuclear cells, and varying numbers of neutrophilic leukocytes, the latter predominating during the later stages of illness. Nasal washings

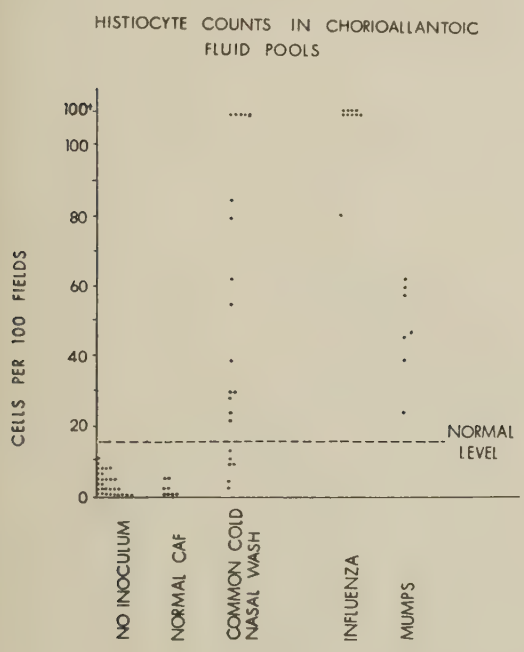

FIG. 1.

TABLE III. Histiocyte Response to Inoculums from 23 Patients with Allergic Rhinitis.*

Date nasal wash		Histiocytes in AF/100 fields
Collected	Inoculated	
9–13–55	9–16–55	11
10– 1	10– 5	4
1	5	3
13	14	5
14	14	4
14	14	8
14	14	3
14	18	0
14	18	10
14	25	6
24	26	10
24	26	5
21	26	7
24	26	4
27	11– 9	5
11– 1	2	4
4	9	15
4	9	4
4	9	3
8	9	3
8	9	4
8	9	4
1– 9–56	1–10–56	15

* Numerous eosinophilic leukocytes noted in smears of nasal effluent.

from 23 cases of acute allergic rhinitis failed to induce histiocytes in excess of the normal limit (Table III). Nasal washings collected during the same period from common cold patients showed in 7 of 8 instances increased numbers of histiocytes in inoculated eggs. (Table IV).

TABLE IV. Histiocyte Response to Inoculums from 8 Cases of Afebrile Rhinitis (Common Cold).

Date nasal wash			Histiocytes in AF/100 fields
Collected	Inoculated	Nasal smear	
10–24–55	10–26–55	Epithelial cells	64
3	11– 9	Mononuclear cells	62
11– 8	9	Rare eosinophiles	100+
11	16	Epithelial cells	17
18	18	No cells	30
21	23	Epithelial cells	70
12– 8	12– 9	*Idem*	75
1– 5–56	1–10–56	"	65

Passage of AF, from eggs in which high counts had been engendered with acute common cold specimens revealed high counts during the succeeding 5 and 6 passages. Thereafter the majority of cell counts declined abruptly to normal levels. Continued passage was accompanied by irregular rises and falls in cell counts. Serial passages of AF from uninoculated eggs induced no significant cell counts in AF. Hemagglutination could not be demonstrated in any of the passaged AF which originated from the acute common cold cases. Bacterial agents could not be demonstrated in any of the fluids by microscopic and by culture technics.

Discussion. The cytological content of AF appears to reflect viral activity, as has been demonstrated in parallel studies for hemagglutinin and histiocyte counts in influenza and mumps-infected allantoic fluids. Both technics were equally sensitive. A similar interpretation might be ascribed to this cytological response by inoculums from acute common cold cases. When the acute nasal washings were treated at 61°C for 30 minutes, this response was negated. Furthermore, the histiocyte response did not occur

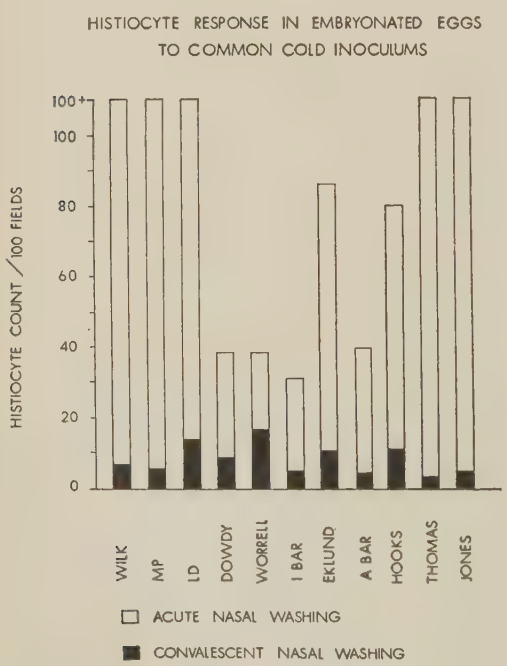

FIG. 2.

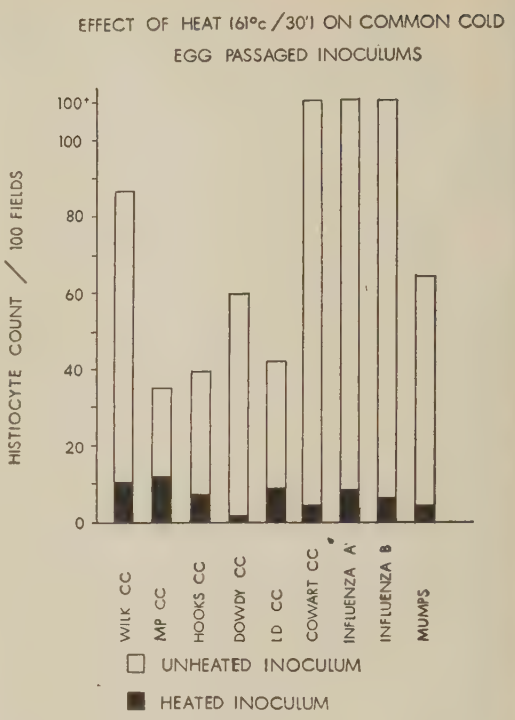

FIG. 3.

with nasal washings collected from the same patients during convalescence. The agent responsible for the histiocyte response may not be the etiological agent of common cold infection; however, its chronological association with the syndrome is suggestive of that possibility. If latent viral agents were responsible for the histiocyte response, they should have been demonstrable in the convalescent nasal washings.

The significance of these results is further supported by the failure of nasal washing from allergic rhinitis cases to engender a significant histiocyte response in AF. Such allergic manifestations were often clinically indistinguishable from the acute cases of common cold. The conditions under which the specimens were collected had no influence on the results, since all specimens were collected and stored under the same conditions (Tables III and IV). The agent(s) do not appear to be influenza-like, since egg passaged material failed to agglutinate chicken erythrocytes.

The histiocyte response is associated with inoculums collected during the acute stage of common cold illness. Although this is a non-

specific response, it might be rendered significant through inhibition by specific antiserums as was demonstrated with influenza virus (Table I). Neutralizing antiserums, prepared in chickens with several strains of common cold agent thus far studied, have demonstrated more than one antigenic strain.

It would be premature to say that even one common cold agent has been adapted to or propagated in the tissues of the embryonated egg. The decline of cytological response with passage gives no support to this proposition. Further studies on optimal passage interval and on tissue susceptibility may provide a means of adapting such agents to avian tissues and the process may be revealed through the cytological response in the AF of inoculated eggs. This would provide a technical advantage over the use of human volunteers for the detection of virus.

Summary. (1) Influenza and mumps viruses induced increased numbers of histiocytes in the allantoic fluids of embryonated eggs. The significance of this response was enhanced by coincident hemagglutinin production and by an interference effect on both responses by specific antiserums. (2) Nasal washings from acute common cold (afebrile rhinitis) patients engendered increased numbers of histiocytes in the allantoic fluid of embryonated eggs. The agent(s) responsible for this cytological response were thermolabile, were not inhibited by penicillin and streptomycin, and produced no detectable growth in bacteriological media. The agent(s) were not demonstrable in nasal washings collected from the same patients during convalescence. (3) Nasal washings from cases of acute allergic rhinitis did not induce increased numbers of histiocytes in the allantoic fluid of inoculated eggs.

1. Kruse, W., *Munch. Med. Wochschr.,* 1914, v61, 1547.

2. Foster, J. B., Jr., *Inf. Dis.,* 1917, v21, 451.

3. Dochez, A. R., Shibley, G. S., and Mills, K. C., *J. Exp. Med.,* 1930, v52, 701.

4. Andrewes, C. H., *Brit. Med. Bull.,* 1953, v9, 206.

5. Andrewes, C. H., Chaproniete, D. M., Gompels, A. E. H., Pereira, H. G., and Roden, A. T., *The Lancet,* 1953, Sept. 12, 546.

6. Kneeland, Y., Jr., Mills, K. C., and Dochez, A. R., PROC. SOC. EXP. BIOL. AND MED., 1936, v35, 213.

7. Pollard, M., and Caplovitz, C. D., *Science,* 1947, v106, 243.

8. Topping, N. H., and Atlas, L. T., *ibid.,* 1947, v106, 636.

9. Ward, T. G., and Proctor, D. F., *Am. J. Hygiene,* 1950, v52, 91.

10. Pollard, M., and Caplovitz, C. D., *Am. J. Hygiene,* 1948, v47, 106.

11. Andrewes, C. H., Lovelock, J. E., and Sommerville, T., *Lancet,* 1951, Jan. 6, 25.

12. Pollard, M., and Diserens, L. T., PROC. SOC. EXP. BIOL. AND MED., 1955, v88, 236.

13. ———, *Fed. Proc.,* 1955, v14, 416.

14. ———, *Bact. Proc.,* 1955, 61.

15. Hirst, G. H., *J. Exp. Med.,* 1942, v76, 195.

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Inhibition by Aminopyrine of Adrenocortical Activation Caused by Pyrogenic Reaction.* (22291)

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Like a number of other stressful stimuli, typhoid vaccine has been shown to cause stimulation of the pituitary-adrenal axis in rats (1). In human subjects, Arendshorst and Falls(2) showed the occurrence of eosinopenia following typhoid vaccine administration, and Rosen(3) and Conn, *et al.*(4) demonstrated increases in urinary 17-ketosteroid and "corticoid" excretion after injection of

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this material. Bliss and his co-workers(5) found that milder febrile stimuli, produced by bacterial pyrogen (Piromen®), caused moderate rises in the levels of plasma 17-OH-corticosteroids.

It seemed that this adrenocortical response to a stimulus might afford a means of studying some of the factors involved in activation of the pituitary-adrenal system. In this study, an attempt was made to learn whether the adrenocortical stimulation depended entirely on the febrile response to typhoid vaccine, or could be produced in the absence of fever. This approach appeared to be of particular interest in view of the studies of Bradley, *et al.*(6), who found that an antipyretic agent inhibited the febrile response to typhoid vaccine, but did not prevent certain renal hemodynamic changes characteristic of the pyrogenic reaction.

Materials and methods. The patients included in this study were 17 adult men and women, in good general health, who were admitted to the Institute of Ophthalmology of the Presbyterian Hospital for treatment of various inflammatory disorders of the eye. Typhoid vaccine (obtained from the Department of Health, City of New York—1 ml contains 1,000 million killed *S. typhosa* organisms, 250 million killed *S. paratyphi* A, and 250 million killed *S. paratyphi* B organisms) was diluted with saline and injected in a standardized manner. 5 to 10 million *S. typhosa* organisms (plus 1.25 to 2.5 million of both *S. paratyphi* A and B) were administered in a single intravenous injection, followed in 2 hours by a similar dose. Rectal temperatures were taken at hourly intervals. Adrenocortical response was assessed by measuring changes in plasma 17-OH-corticosteroid levels, determined according to the method of Silber and Porter(7) as previously described(8). Control blood specimens were obtained, and additional specimens were drawn at intervals following administration of typhoid vaccine (Table I). No rise in plasma 17-OH-corticosteroid level was regarded as significant unless values exceeded the upper limit of normal. Normal limits in this laboratory are 4-28 μg per 100 ml plasma.

TABLE I. Effect of Intravenous Typhoid Vaccine on Levels of Plasma 17-OH-Corticosteroids. Time in hr after 2nd vaccine injection.

Patient	Max temp., °F	Plasma 17-OH-corticosteroids, γ % Control	2 hr	4 hr	6 hr
1	103.8	18	43	34	
	103.8	28		42	40
2	104.8	15	59	30	
	102.4	13	32		
3	103.8	17	40	35	16
	103.4	22		32	
4	102.6	20	40		
5	102.8	13		30	
6	105.6	14	53	36	
7	104.2	8	28	29	
	105.6	13	46	34	16
8	103.8	7	32		
9	103.6	17	36		42
	104.0	19	37		13
	104.8	18		35	
10	103.6	17		35	
	102.6	24			24
11	104.0	20	43	45	
12	102.6	18	33	22	
	102.4	14	38		
13	104.4	17	36		
	103.4	26		27	
14	102.6	16	36		
15	102.4	14	29	17	

In 7 of the patients, the anti-pyretic, aminopyrine was given over a 24-hour period in a dose of 0.6 g every 4 hours. Immediately following the last dose of aminopyrine, typhoid vaccine was administered according to the schedule outlined above, with blood samples being obtained in the same manner.

Results. 1. *Effect of typhoid vaccine alone.* In 15 patients, typhoid therapy was given on 24 separate occasions. In all 24 instances, there was a sharp rise in temperature to 102.4-105.6°F, occurring within 2 to 5 hours after the second dose of vaccine. The control 17-OH-corticosteroid values fell within the normal range (Table I). In 22 of the 24 experiments, plasma 17-OH-corticosteroid levels rose, within 2 to 6 hours after the second dose, to values above the normal which ranged from 29 to 59 μg per 100 ml plasma. These levels were comparable to those attained in normal subjects after intravenous infusion of 25 mg ACTH(9). In 4 of the patients, in whom studies were made of diurnal variations in levels of circulating 17-OH-

corticosteroids, such abnormally high values were not found. In these patients, corticosteroid levels fluctuated within the normal range. In 4 additional instances, typhoid vaccine failed to produce a rise in temperature above 101°F, and the febrile response was delayed. In these cases, plasma corticosteroid levels did not rise above the upper limit of normal.

2. *Effect of aminopyrine.* In all 7 patients treated with aminopyrine, injection of typhoid vaccine failed to cause pyrexia above 100.4°F. In 6 of the patients, there were no significant rises in plasma 17-OH-corticosteroid levels, values remaining within normal limits (Table II). In the seventh (patient #8, Table II), plasma 17-OH-corticosteroid levels rose from 20 to 28 μg per 100 ml, the upper limit of normal. It was noteworthy that in all 7 subjects, constitutional symptoms (malaise, weakness, nausea, chilly sensations) occurred despite the absence of fever. Failure of plasma 17-OH-corticosteroid levels to rise in the aminopyrine-treated subjects did not seem attributable to refractoriness to the pyrogenic effect of the vaccine. As can be seen in Fig. 1, repeat administration of comparable doses of vaccine in the 3 patients restudied without aminopyrine, again caused rises in temperature, associated with an increase in plasma 17-OH-corticosteroid values.

3. *Effect of central nervous system depressants.* (a) Barbiturate. In a single subject, 1 g of Amobarbital sodium (Na amytal) was administered in divided doses before and during standard vaccine injection. The temperature rose to 105.6°F within 3 hours after the second dose of vaccine. Plasma 17-OH-corticosteroid rise was not inhibited, rising from a control level of 13, to 43 μg per 100 ml.

(b) 10-(γ-dimethylaminopropyl)-2-chlorophenothiazine (Chlorpromazine). This broadly-acting agent, chosen because of its depressant effect on hypothalamic centers(10), was administered to 8 patients in doses up to 250 mg, before and during typhoid vaccine therapy. In 6 instances, plasma 17-OH-corticosteroid rises occurred in a manner comparable to that shown in Table I. In 2 cases, corticosteroid rises did not occur, but in both instances, febrile response was delayed and was of only moderate degree.

4. *Controls.* (a) To rule out the possibility that the aminopyrine suppression of plasma corticosteroid rise could be due to blockade of adrenocortical response to endogenous ACTH, a single normal male was treated for 24 hours with aminopyrine (0.6 g every 4 hours), and then subjected to a standardized ACTH test. Plasma 17-OH-corticosteroid levels rose from 10 to 45 μg per 100 ml, a response well within the limits of normal(9). (b) To exclude a direct inhibitory effect of aminopyrine on pituitary release of ACTH, a normal subject was given an insulin tolerance test (0.15 unit of standard insulin per kilogram body weight administered intravenously). Plasma 17-OH-corticosteroid levels rose from 20 to 28 μg per 100 ml(5). After treatment for 24 hours with aminopyrine, 3.6 g, repeat insulin administration was again associated with a rise in plasma corticosteroid value, from 6 to 30 μg per 100 ml. (On 2 different days, diurnal variation in plasma corticosteroid levels in this patient showed a definite fall in values.)

TABLE II. Effect of Aminopyrine on Plasma 17-OH-Corticosteroid Response to Intravenous Typhoid Vaccine. Time in hr after 2nd vaccine injection. (Patient numbers correspond to those in Table I.)

Patient	Max temp., °F	Plasma 17-OH-corticosteroids, γ % Control	2 hr	4 hr	6 hr
1	99.4	25	24		13
2	99.6	14	17		11
3	100.4	24	22	24	
8	99.6	20	28		
14	98.6	14	19		
16	99.0	27	27		
17	98.4	28	20		

Discussion. The rise of plasma 17-OH-corticosteroids to levels above the normal following typhoid vaccine injection confirms the fact that this agent is capable of producing adrenocortical activation in the human subject(2-5). Blockade of the febrile response to the vaccine by aminopyrine inhibits this plasma corticosteroid increase. The inference is that the fever *per se* is the essential factor in bringing about activation of the pituitary-adrenal system. Presumably, other

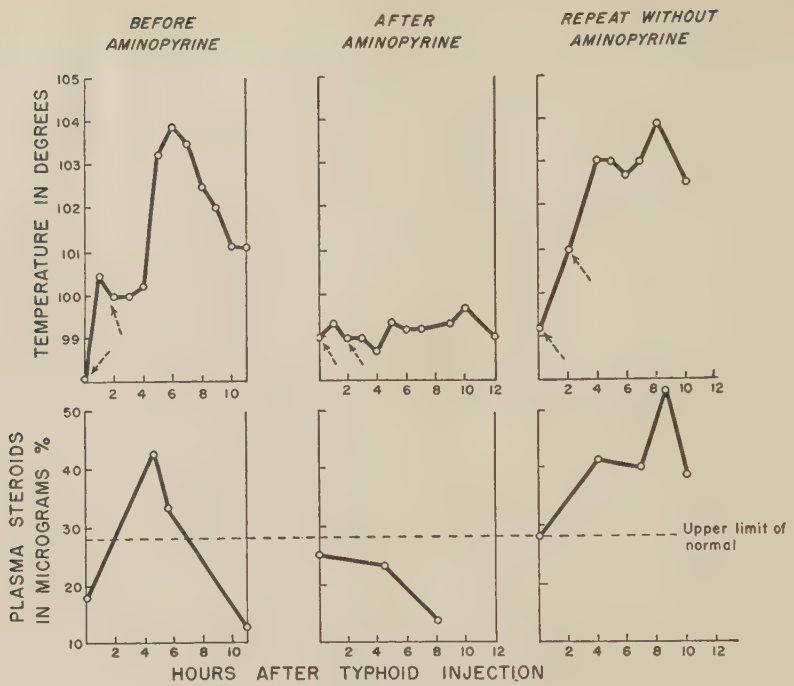

FIG. 1. Febrile and plasma 17-OH-corticosteroid response to intravenous typhoid vaccine injection (patient #1, Table I). The 3 series of vaccine injections were performed at intervals of 3 days. Arrows indicate times of injection of vaccine.

noxious effects of the vaccine which are not prevented by an antipyretic agent(6), are not effective in activating the adrenal cortex, as measured by this index of response. Furthermore, other central nervous system depressants, in the doses used, failed to inhibit plasma corticosteroid rises, unless the febrile response was also partially blocked.

The inhibitory effect of aminopyrine did not appear to act directly on the pituitary-adrenal system. The drug did not interfere with the adrenocortical response to administered ACTH, nor did it inhibit the pituitary-adrenal reaction to a non-febrile stimulus (insulin hypoglycemia).

Summary. Intravenous administration of typhoid vaccine to human subjects produced adrenocortical activation, as measured by increases in levels of circulating 17-OH-corticosteroids. Suppression of the pyrogenic effect of the vaccine by aminopyrine prevented adrenocortical response, without blocking certain other systemic reactions.

1. Sayers, G., and Sayers, M. A., *Ann. N. Y. Acad. Sci.,* 1949, v50, 522.

2. Arendshorst, W., and Falls, H. F., *Arch. Ophthalmol.,* 1950, v44, 635.

3. Rosen, D. A., *Am. J. Ophthalmol.,* 1952, v35, 1783.

4. Conn, J. W., Fajans, S. S., Louis, L. H., Seltzer, H. S., and Kaine, H. D., *Recent Prog. Hormone Res.,* 1954, v10, 471.

5. Bliss, E. L., Migeon, C. J., Eik-Nes, K., Sandberg, A. A., and Samuels, L. T., *Metabolism,* 1954, v3, 493.

6. Bradley, S. E., Chasis, H., Goldring, W., and Smith, H. W., *J. Clin. Invest.,* 1945, v24, 749.

7. Silber, R. H., and Porter, C. C., *J. Biol. Chem.,* 1954, v210, 923.

8. Wallace, E. Z., Christy, N. P., and Jailer, J. W., *J. Clin. Endocrinol. and Metab.,* 1955, v15, 1073.

9. Christy, N. P., Wallace, E. Z., and Jailer, J. W., *J. Clin. Invest.,* 1955, v34, 899.

10. Aron, E., *Anesth. et Analg.,* Paris, 1954, v11, 399.

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Further Studies of Interrelationship Between Xanthine Oxidase and Influenzal Pneumonia in Mice.* (22292)

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It has been previously reported(1) that an increase in xanthine oxidase occurs in the lungs of mice infected with PR8, Type A influenza virus. In order to define further the interrelationship between the increased levels of enzyme and virus proliferation additional experiments have been carried out. Levels of the *in vitro* activity of xanthine oxidase in mouse lung infected with influenza virus have been compared with those of the same tissue either infected or injured with: (a) Nigg pneumonitis virus, (b) Newcastle disease virus, (c) *Klebsiella pneumoniae,* and (d) toxic chemicals, fats, Friedlander's polysaccharide and leukotaxine. The results indicate that although the increase in xanthine oxidase is not directly related to influenza virus proliferation *per se,* the enzyme may be concerned in some way with the pneumonic process in mouse lung.

Methods. Four to 6 weeks old albino Swiss mice of the Webster strain were employed. Xanthine oxidase assays on homogenized mouse lung tissue were carried out in a conventional Warburg apparatus as described previously(1). The values reported represent the oxygen uptake, stimulated by addition of substrate, of 20 mg tissue (dry weight equivalent) per 20 minutes; in each experiment the oxygen uptake representing the endogenous respiration has been subtracted from the recorded values. Hemagglutination titrations were performed employing a pattern end point (2). In determining the virus infectivity titers, equal volumes of a sample of homogenate from all the animals assayed at a given time were pooled, serial 10-fold dilutions prepared and 4, 10-day chick embryos inoculated with 0.2 ml of each dilution. After 48 hours incubation at 35°C, samples of allantoic fluid were removed and tested for the presence of virus by the spot plate hemagglutination technic(3). The ID_{50} titers were calculated according to the method of Reed and Muench (4). The lungs from each animal were examined and the pulmonary consolidation scored according to the method of Horsfall (5).

Results. The results of an experiment with a mouse adapted strain of PR8, Type A influenza virus are presented in Table I. Mice were infected via the intranasal route with 4,000 LD_{50} doses of stock virus. The day-to-day change in enzyme level together with the change in virus level as determined by hemagglutination titers for chick-embryos were determined on combined aliquots of tissue from each day's experiment; these results are directly comparable to the recorded averages of enzyme activity. Levels of enzyme activity are roughly paralleled by virus titers.

Consolidation is somewhat slower than is the increase in enzyme activity in developing; the presence of gross lesions is not essential for increased xanthine oxidase activity although the maximal levels of enzyme are found in partially consolidated tissue. A lethal dose of virus was employed in this experiment and at the end of 100 hours all the animals were dead. When sub-lethal amounts of virus were employed comparable results were obtained, *i.e.,* the development of enzyme levels and virus titers roughly paralleled each other although the levels reached were lower and slower in developing than in lethally infected mice.

An experiment showing the changes in xanthine oxidase in mouse lung following infection with Nigg pneumonitis virus (Greb

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TABLE I. Correlation of Changes in Virus Concentration and in Xanthine Oxidase Activity in Mouse Lung Infected with PR8 Influenza Virus.

Time (hr)†	Group 1* E.A.‡	H.T.§	C.‖	Group 2 E.A.	H.T.	C.	Group 3 E.A.	H.T.	C.	Avg E.A.	H.T.	EID_{50}
0	2.3			2.5			2.3			2.4		
24	6.9	160	0	5.3	160	+	4.9	160	0	5.7	160	$10^{-7.75}$
			0			0			0			
48	8.5	320	0	7.2	320	+	5.9	320	0	7.2	320	$10^{-8.5}$
			0			+			+			
72	13.2	320	2+	9.8	320	4+	8.0	160	3+	10.3	266	10^{-9}
			+			2+			2+			
96	12.0	80	3+	4.8	320	3+	3.9	80	2+	6.9	240	$10^{-7.5}$
			+			2+			+			

* Group of 10 mice, 2 chosen at random and pooled for examination at indicated time.
† No. of hours between initial infection and titration.
‡ Enzyme activity: μl O_2 uptake/20 min./20 mg dry lung. Substrate: 10 μM hypoxanthine/flask.
§ Hemagglutinin titer: Highest dilution of virus showing complete hemagglutination.
‖ Amount of consolidation determined by gross macroscopic examination and scored on a 0-5 point basis.
EID_{50}—50% embryo infectivity end point.

strain) is recorded in Table II. The animals were infected intranasally with 10-100 lethal doses of virus. Although the titer of virus in the tissue examined for enzyme activity was not determined, the presence of virus was established by mouse inoculation. In comparison to the PR8 infection, a somewhat greater level of enzyme activity was reached on each successive day reaching a peak value of 12.4 on the fourth day. Only 3 of the animals survived to the fifth day; the value obtained for the enzyme activity of these lungs was 11.4 which may have indicated a leveling off of activity.

Table III shows the changes in xanthine oxidase in mouse lung following the administration of Newcastle disease virus (NDV). The mice were given 0.05 ml of infected allantoic fluid (ID_{50} titer for eggs of $10^{-7.6}$) by the intranasal route. There was a sharp rise in enzyme activity almost reaching a peak level within 24 hours and remaining relatively constant through the fourth day, while the macroscopic consolidation developed gradually over a three day period.

For comparative purposes, studies were made of the effect of a pulmonary bacterial infection on xanthine oxidase. Virulent *Klebsiella pneumoniae*, when introduced via the intranasal route, produced a rapidly fatal pneumonitis. Within 48 hours the lungs were almost completely consolidated, and the ani-

TABLE II. Change in Levels of Xanthine Oxidase Activity in Mouse Lung after Infection with NIGG Pneumonitis Virus.

Time (hr)†	Group 1* E.A.‡	C.§	Group 2 E.A.	C.	Group 3 E.A.	C.	Avg E.A.
0	2.6		2.0		2.1		2.2
24	6.4	+	5.6	+	3.8	+	5.26
		—		+		—	
48	11.4	4+	8.1	3+	8.0	3+	9.16
		+		2+		+	
72	11.0	4+	10.7	3+	10.0	3+	10.56
		2+		3+		3+	
96	14.6	3+	11.8	4+	10.9	4+	12.43
		3+		3+		3+	

* Group of 10 mice, 2 chosen at random and pooled for examination at indicated time.
† No. of hours between initial injection and assay.
‡ Enzyme activity: μl O_2/20 min./20 mg dry lung. Substrate: 10 μM hypoxanthine/flask.
§ Consolidation.

TABLE III. Change in Levels of Xanthine Oxidase Activity in Mouse Lung after Administration of Newcastle Disease Virus.

Time (hr)†	Group 1* E.A.‡	C.§	Group 2 E.A.	C.	Group 3 E.A.	C.	Avg E.A.
0	2.6		2.0		2.1		2.2
24	10.0	0	8.3	0	6.5	+	8.26
		+		+		+	
48	9.0	4+	8.1	+	7.7	+	8.26
		3+		0		+	
72	11.8	5+	8.9	2+	7.3	4+	9.33
		0		+		4+	
96	11.6	+	9.4	5+	7.0	+	9.33
		+		3+		+	

* Group of 10 mice, 2 chosen at random and pooled for examination at indicated times.
† No. of hours between initial inoculation and assay.
‡ Enzyme activity: μl O_2/20 min./20 mg dry lung. Substrate: 10 μM hypoxanthine/flask.
§ Consolidation.

mals seldom survived a lethal dose beyond this time. Enzyme assays showed that the level of xanthine oxidase was increased concomitant with the progressing *K. pneumoniae* infection: Values of 4.0, 5.4, and 6.5 (μl O_2 uptake/20 minutes/20 mg dry lung) at 24 hours and 5.4, 5.6, and 6.7 at 48 hours were obtained in a typical experiment. These enzyme levels, when compared with the levels of lungs infected with influenza virus are comparable at the same time intervals; however, the maximal levels of enzyme in the influenza lungs at the end of 96 hours, when the lesions are more comparable, are about two times these values. That the enzyme activity was not due to the bacteria present in the homogenate was shown by testing the organisms for xanthine oxidase activity. The strain of *K. pneumoniae* employed in these experiments showed almost no activity, and efforts to produce an "adaptive" type of this enzyme were not successful. The results indicate that infection of mouse lung with *K. pneumoniae* produces a severe pneumonitis and gives rise to an increase in xanthine oxidase activity.

The following compounds were employed in attempts to produce a sterile pneumonia in mice: dilute ammonia, Friedlander's polysaccharide, olive oil, starch, heparin, leukotaxine, serum and turpentine. Intranasal, intratracheal and intrapleural routes of inoculation were tried. In no instance was a pneumonic process produced equal in magnitude to that produced by virus or *K. pneumoniae*. In 10-25% of the animals one lobe, or perhaps 2 lobes, showed some consolidation; xanthine oxidase was not increased in these lungs. The most successful procedure was that of intrapleural injection of turpentine; at the end of 96 hours some of these lungs showed a moderate amount of consolidation and a concomitant increase in xanthine oxidase of about the same magnitude as the 48-hour influenza lung. Therefore, it is apparent that the increase in xanthine oxidase can be elicited in the absence of an infectious process and may be the result of an inflammatory reaction.

In order to determine if basic similarities existed in the pathology of the lesions produced by these various agents, microscopic sections were examined. Sections of influenza lungs showed diffuse interstitial pneumonitis with a mononuclear infiltrate causing a thickening of septal walls. Necrosis and desquamation of the epithelial lining of the bronchi and bronchioles were noted. The lesions produced by NDV were very similar in character although less severe in degree. Sections of the lesions produced by *K. pneumoniae* and by Nigg pneumonitis virus showed severe bronchopneumonia with diffuse areas of cellular infiltrate, predominantly polymorphonuclear leucocytes. There was associated distortion and destruction of lung architecture. The mucosae and walls of the bronchi and bronchioli appeared to be relatively uninvolved although the lumina were plugged with exudate. A correlation between lesions with characteristic infiltrates and xanthine oxidase activity was not found.

Discussion. In mouse lungs infected with the viruses of influenza and Nigg pneumonitis, a fundamental relationship between purine catabolism and virus synthesis is suggested by an increase in xanthine oxidase activity which occurs during the phase of rapid increase in virus concentration and precedes the development of maximal macroscopic and microscopic lesions. The fact that a similar change occurs after NDV which does not multiply in mouse lung(6,7) might contradict such a hypothetical relationship. On the other hand, it is conceivable that a change in purine metabolism is initiated by invasion of the host cell by viral particles whether or not subsequent multiplication of the virus takes place. Comparative studies of other types of injury to mouse lung indicate that infection with *K. pneumoniae,* and the intrapleural injection of turpentine also result in significant increases in xanthine oxidase activity. These latter findings negate the intriguing possibility that xanthine oxidase is concerned with a phase of nucleic acid metabolism intimately related to viral synthesis. The fact that a variety of microorganisms capable of producing severe and rapidly fatal pneumonias all elicit a rapid increase in xanthine oxidase activity remains of some interest. One may ask whether or not this represents a biochemical disturbance of host-cell metabolism which contributes to the over-all disease process.

Conclusion. Previous observations that xanthine oxidase increases in mouse lung concomitant with influenza virus infection have been confirmed and extended. Comparable increases in xanthine oxidase also occur in murine pneumonitis produced by Nigg pneumonitis virus and by NDV. Comparative studies with other types of infection and injury indicate that infection with *K. pneumoniae* and the intrapleural injection of turpentine also result in significant increases in xanthine oxidase activity in mouse lung thus suggesting that an increase in the level of enzyme is closely associated with injury and death to lung tissue.

1. Sellers, M. I., and Jann, J. G., PROC. SOC. EXP. BIOL. AND MED., 1954, v86, 205.
2. Salk, J. E., *J. Immunol.,* 1944, v49, 87.
3. Rasmussen, A. F., Jr., Stokes, J. C., and Smadel, J. E., *Am. J. Hyg.,* 1945, v42, 45.
4. Reed, L. J., and Muench, H., *ibid.,* 1938, v27, 493.
5. Horsfall, F. L., Jr., *J. Exp. Med.,* 1939, v70, 209.
6. Ginsberg, H. S., *ibid.,* 1951, v94, 191.
7. Davenport, F. M., *J. Immunol.,* 1952, v69, 461.

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Fluorescent Antibody Studies of Demyelination in Canine Distemper.* (22293)

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The literature on central nervous system lesions in canine distemper has been adequately reviewed by Winqvist(1). Demyelination and glial cell proliferation are the outstanding changes while vascular hyperplasia and perivascular infiltrations occur less frequently. The cerebellum, pons and medulla are the principal sites of involvement. No attempt has been made to correlate the demyelination with localization of the virus. This report describes the demyelination process as it occurs in the cerebellum, and by means of the fluorescent antibody technic(2) to demonstrate the sites of viral concentration.

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Methods. Tissues for histopathologic study were obtained at autopsy from naturally occurring cases of distemper. All the dogs had typical distemper inclusion bodies either in urinary bladder epithelium or in astrocytes of the cerebellum. Blocks of cerebellum were fixed in 10% formalin, Zenker's and ammonium bromide-formalin mixtures. Hematoxylin and eosin, Weil, Bodian protargol, pyridine silver, cresyl violet, Feulgen Mallory phosphotungstic acid, Cajal gold sublimate and oil red O. B. staining methods were employed. The fluorescent antibody technic is based on the fact that fluorescein can be conjugated with antibody molecules without loss of activity. When tissue sections containing antigen specific for the antibody solution are flooded with such fluorescein-antibody conjugate, the antibody combines with the antigen. After the excess conjugate is rinsed off, the antibody is visible under a fluorescence microscope by virtue of its fluorescent yellow-green label. *Hyperimmune canine distemper* serum was conjugated to fluorescein isocyanate according to the method of Coons and Kaplan(2). Conjugates with greater antibody titer were obtained with freshly prepared isocyanate than with isocyanate which had been previously prepared and stored. Tissues for fluorescent staining were obtained from clinical cases of distemper immediately after death. Blocks of cerebellum were placed in test tubes and quickly frozen by immersion in a bath of dry ice and alcohol. They were stored at –20°C until needed. Frozen sections were cut from these blocks at 15μ and mounted directly on slides without floating on water. Sections were dried on the slides by placing them in an incubator at 37°C for one hour. No chemical fixatives were employed. The sections were stained 30 minutes in fluorescein-antibody conjugate and then rinsed off with buffered saline, pH 9, and gently washed in fresh buffered saline for 10 minutes. Slides were wiped dry except for the section and mounted in glycerol-saline(9:1). Sections were examined in the dark with a fluorescence microscope (3). The light source was a Leitz, 15 ampere, carbon arc. Specific fluorescence appeared brilliantly yellow-green against a dark background. Fluorescent structures were identified by floating off the coverslip of the slide, counterstaining with a color dye and examining the same field under white light (3).

The *conjugate* used in this study was tested for antibody content by means of an *in ovo* neutralization test(4). The first sample had 1:630 titer and its deactivated blank, which was heated to 65°C for one hour, tested 1:250. The second sample had a titer of 1:160 and its blank, which was heated to 95°C for one hour, was negative. The difference in titers between the two samples was due to variations in quantity of virus used in the test and did not indicate a drop in antibody content in the conjugate(4). Conjugate was tested for specificity by staining smears of urinary bladder epithelium from dogs with distemper. Inclusion bodies in bladder epithelium show specific fluorescence when stained with distemper fluorescent antibody(3). *Slide controls* consisted of 1) staining brain tissues of normal 7-week-old puppies in the same manner as tissues from infected dogs, and 2) inhibiting the antigen in the tissue sections by treating with unconjugated distemper antiserum for 30 minutes prior to staining with conjugate.

Results. Weil stains of the cerebellum showed patchy areas of demyelination in the medullary white matter (Fig. 1). Early lesions appeared as empty spaces or holes in the white matter where there had been focal loss of myelin in 4 or 5 fibers. With loss of myelin, edema fluid collected in these foci, forced demyelinated axis cylinders to the periphery and compressed adjacent myelinated fibers.

When many holes formed in an area they coalesced into larger, spongy areas of demyelination (*Lückenfelder*). At the same time glial proliferation was noted. This occurred in response to disintegrated myelin for the majority of cells were microglial elements containing droplets of myelin (Fig. 2). Ameboid astrocytes were also present in the demyelinated areas. Glial scars were never observed.

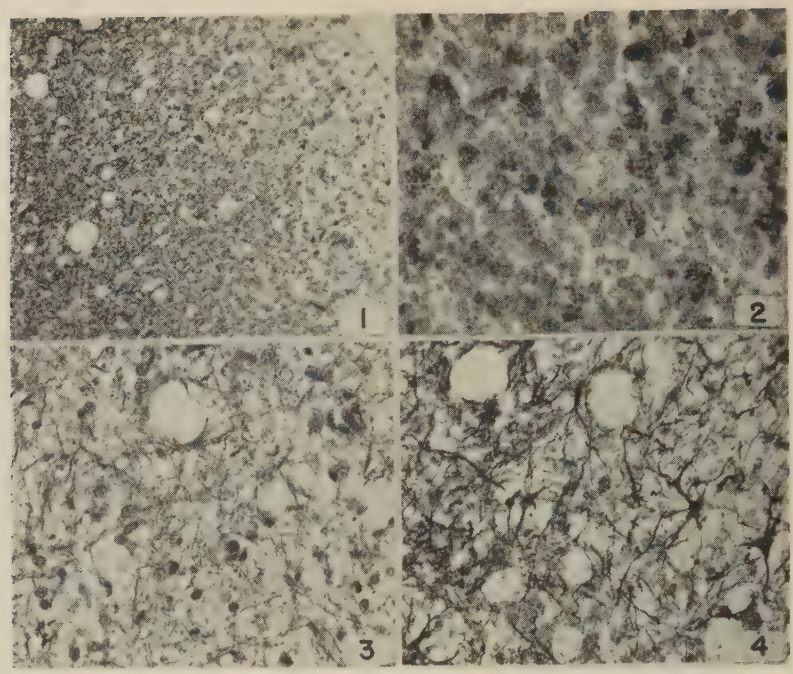

FIG. 1. Demyelination of cerebellar white matter in canine distemper. Weil stain. × 225.

FIG. 2. Microglial phagocytes filled with myelin droplets in an area of demyelination. Oil red O. B. stain. × 450.

FIG. 3. Axis cylinders in area of demyelination. Fibers show separation but no loss in continuity. Bodian protargol. × 450.

FIG. 4. Astrocytes in area of demyelination. Processes are intact although pushed apart by edema fluid. Cajal gold sublimate. × 450.

Axis cylinders were seldom involved although cylinders passing through severely demyelinated areas were stained poorly and separated by the edema fluid (Fig. 3).

The majority of fibrous and ameboid astrocytes exhibited degenerative nuclear changes. This was not limited to astrocytes in demyelinated areas, but was also found in astrocytes at a distance. Astrocytic processes were not involved (Fig. 4). The nuclei of the degenerated astrocytes appeared swollen and pale and often contained one or more acidophilic inclusion bodies (Fig. 5). Nuclear chromatin was marginated in these cells. Feulgen reactions were weakly positive in the degenerated astrocyte nuclei and negative in the inclusion bodies. Oligodendroglia showed some nuclear degeneration, but fewer cells were involved. Neurons were normal except for those in severely demyelinated areas which showed chromatolysis.

Inflammatory cells were usually absent, but in severely demyelinated areas perivascular inflammatory cells were sometimes noted. These consisted of plasma cells, lymphocytes and macrophages and were restricted to the Virchow-Robin space. A few sections exhibited proliferation of blood vessels in advanced areas of demyelination.

Sections stained with fluorescein-antibody conjugate showed specific fluorescence in the cerebellar white matter (Fig. 6). Fluorescence in the cerebellar medullary lamellae was especially remarkable because of the contrast of the non-fluorescent cortex (Fig. 7). Specific yellow-green fluorescence appeared to be localized principally in astrocyte nuclei although it was difficult to tell astrocytes from other glial cells. It was impossible to tell whether or not inclusion bodies were fluorescing. Fluorescence was not limited to cells in demyelinated areas, but involved cells throughout the white matter. Myelin sheaths, neurons and axis cylinders showed no specific fluorescence.

Control slides reacted negatively. Sections

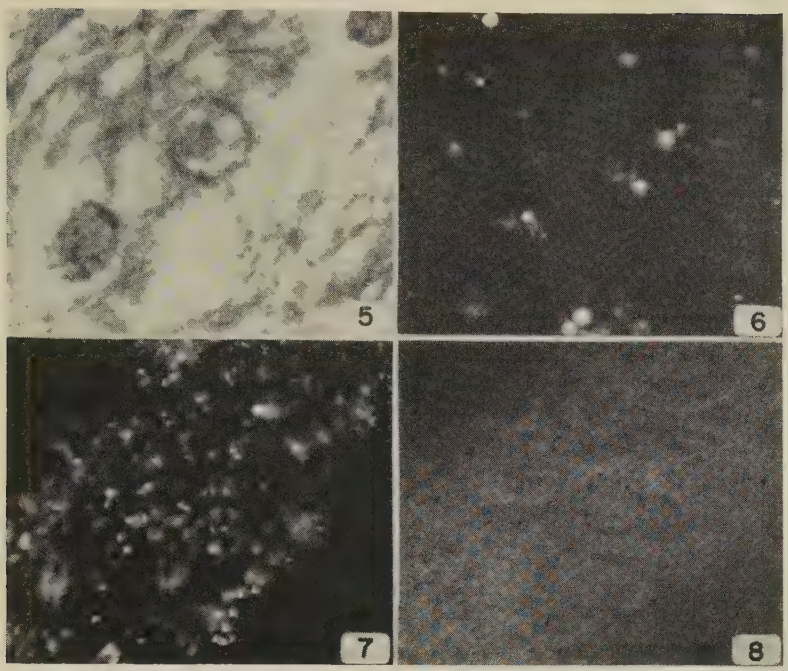

FIG. 5. In center is an inclusion body in nucleus of an astrocyte. Hematoxylin and eosin. × 600.

FIG. 6. Localization of viral antigen in glial nuclei of cerebellar white matter. Fluorescent antibody technic. × 450.

FIG. 7. Cerebellar lamella of dog with distemper showing fluorescent staining of glial cells. Note absence of fluorescence in adjacent cortex. Fluorescent antibody technic. × 400.

FIG. 8. Same area as in Fig. 7 from a normal dog. This section was treated identically. Note absence of specific fluorescence in cerebellar lamella. Fluorescent antibody technic. × 450.

from normal dogs showed no specific fluorescence (Fig. 8). In pathological specimens staining was markedly reduced in tissues treated with normal antiserum before fluorescent antibody exposure.

Discussion. Demyelination has seldom been listed among the lesions produced by viruses infecting the nervous system. Viruses have not been considered seriously as possible etiological agents of the various demyelinating maladies. There is no experimental support for the belief that the post-vaccinal type of encephalomyelitis results from direct action of the virus(5). In the case of nervous distemper, however, recent studies point to the distemper virus as the agent responsible for the demyelination and the presence of nuclear inclusions in the glial cells is evidence of the presence of the virus(6). However, the mechanism of demyelination has not been explained.

Fluorescent antibody studies of distemper indicate that neuroglial nuclei are invaded by the virus and that the astroglia are primarily involved. There is good correlation between astrocytic nuclear degeneration as observed by conventional staining methods and localization of viral antigen as demonstrated with fluorescent antibody. Demyelination in distemper does not appear to be associated directly with neuronal or axis cylinder degeneration as these structures do not show specific fluorescent staining and exhibit few degenerative changes. It appears that demyelination in this disease is associated with viral invasion and degeneration of glia, especially astroglia although this relationship cannot be explained. Other than the possible association of oligodendroglia with the production and maintenance of myelin(7), no other relationship between glial cells and myelination is known.

When sufficient myelin is broken down, microglial phagocytes invade the area and

ameboid astrocytes are evident. Perivascular inflammatory reaction is not a constant finding in this disease, but when it occurs it is probably a secondary reaction in response to disintegration of myelin(8).

Summary. Fluorescent antibody studies show that the virus of canine distemper is localized in astrocytic and possibly other glial nuclei. The astrocytes exhibit degenerative changes consistent with viral invasion. Astrocytic involvement appears to be followed by demyelination although this causative relationship cannot be explained.

1. Winqvist, G., *Nord. Vet.-med.,* 1950, v2, 367.

2. Coons, A. H., and Kaplan, M. H., *J. Exp. Med.,* 1950, v91, 1.

3. Moulton, J. E., and Brown, C. H., PROC. SOC. EXP. BIOL. AND MED., 1954, v86, 99.

4. Baker, G. A., Gorham, J. R., and Leader, R. W., *Am. J. Vet. Res.,* 1954, v15, 102.

5. Hurst, E. W., *Brain,* 1944, v67, 103.

6. Cooke, B. T., and Melvin, P., *Austral. J. Exp. Biol. Med. Sci.,* 1943, v21, 115.

7. Penfield, W., *Cytology and Cellular Pathology of the Nervous System,* Paul B. Hoeber, Inc., New York, 1932, v2, 1033.

8. ———, *ibid.,* 1932, v3, 1221.

Received January 30, 1956. P.S.E.B.M., 1956, v91.

Monolayer Cultures of Trypsinized Monkey Kidney Cells in Synthetic Medium. Application to Poliovirus Synthesis.* (22294)

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The outgrowth of monkey kidney cells from suspensions of trypsinized tissue has always been found to require substantial amounts of serum in the medium. The necessity for adding serum has precluded many kinds of studies and has restricted the kind of cultures available for study. A simple synthetic medium is described which, in the absence of any added protein, supports outgrowth of monkey kidney cell suspensions. After outgrowth in this medium, the cells support synthesis of poliovirus and may be used for physiological studies under defined conditions in the absence of serum.

Methods. Cell suspensions. Trypsinization of kidneys taken from rhesus monkeys was done by a modification(1) of the Youngner procedure(2). The cells were harvested from the trypsin fluid by centrifugation at 200 rpm for 30 minutes, resuspended in 500 x their volume of Basic Salt #2 without bicarbonate (as described in Table I), and recentrifuged. The washed cells, resuspended in 100-200 ml of synthetic medium, were filtered through 2 layers of cheese cloth. A count of those cells showing both nuclei and attached cytoplasm was made by staining with 0.1% crystal violet-0.1 M citric acid, and the suspensions were diluted to 350,000 cells per ml. Amounts of 0.5 ml were seeded into 16 x 150 mm test tubes, 7 ml into 2 oz and 12 ml into 4 oz prescription bottles, and 80-100 ml into Roux flasks. These amounts are also in the optimal range for cells suspended in the medium which contains serum (1). In experiments where outgrowth in the synthetic medium was to be compared to outgrowth in a serum-containing medium, the suspension in PBS was divided into 2 portions and the second half after recentrifuging was diluted into the serum-containing medium. The serum-containing, "complete" medium (called "M") used for comparison was the 0.5% lactalbumin hydrolysate-2% calf serum medium in Hanks' salt solution which has been used extensively in this laboratory(3).

Virus titrations. These were made by the

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TABLE I. Synthetic Medium (SM-1) for Outgrowth of Monkey Kidney Cell Suspensions.

Basic salt sol. (B.S. #2)*		Trace elements†	
	g/l		M × 10^{-6}/l
NaCl	8.0	$ZnSO_4$	3
KCl	.4	$Fe(NO_3)_3$	2
$MgSO_4$ 7 H_2O	.1	$CoCl_2$	.2
$MgCl$ 6 H_2O	.1	$MnCl_2$	.5
Na_2HPO_4	.08	$CuSO_4$	.4
NaH_2PO_4	.02		
$CaCl_2$	.42		
$NaHCO_3$	.78		
Glucose	2.0		

Organic supplements‡		Supplementary buffer§
	mg/l	
l-cysteine • HCl	30	Tris (hydroxymethyl
l-isoleucine	60	aminomethane)
d-ribose	30	8 × 10^{-3} M, pH 7.6

* Made in 10 × concentrated, combined stock without $CaCl_2$ and $NaHCO_3$. Sterilized by filtration. $CaCl_2$ is made at 100 × concentrated; $NaHCO_3$ is made at 100 × concentrated. Glucose in 20% stock solution, sterilized by filtration.

† Held as separate stocks, 0.05% concentrations. Sterilized by filtration.

‡ Stocks 100 × concentrated. Sterilized by filtration. May be held as combined stock 40 × concentrated in H_2O.

§ Conveniently stored as 2 M stock adjusted to pH 7.6 with HCl. May be sterilized by autoclaving at 15 lb for 15 min.

Dulbecco-Vogt plaque method on cultures grown out in the indicated medium in 4 oz prescription bottles(3). The culture fluid was decanted, and the inoculum of virus was added from the same pipette to a series of 12-20 bottles. After incubation for 1 hr at 37°C, bottles of each series were randomly selected and overlaid with calf serum agar overlay, "C.S.", or with amino acid supplement agar overlay "AAS", made as follows: *C.S.*: Agar (2.7 g/60 ml), 33%; calf serum, 2.5%; neutral red (1/1000 of 80% dye), 1.8%; Earle's salt solution 10 x concentrated, 10%; H_2O, 50%; $NaHCO_3$ (58 g/50 ml solution), 2.05%. *AAS*: (solutions as in C.S. overlay) Agar, 33%; basic salt #2 (Table I) 2 x concentrated, 50%; H_2O, 8%; neutral red, 1.8%; amino acid supplement 40 x concentrated, 2.5%; Na_2HPO_4 (5% solution), 0.28%; $NaHCO_3$, 3.8%. Solutions added in order given.

Experimental. The synthetic medium, SM-1, which has been used routinely for outgrowth of monkey kidney cell suspensions, is described in Table I. It consists of a salt solution containing a higher concentration of calcium than is widely used; trace elements; and the organic constituents, cysteine, isoleucine, and d-ribose. The buffering capacity of the medium is increased by the addition of 8 x 10^{-3} M Tris (hydroxymethyl aminomethane). It may be supplemented with penicillin and streptomycin if desirable. The medium is stable for at least one week at 4°C, and is not used once a precipitate forms on standing. The medium cannot be frozen.

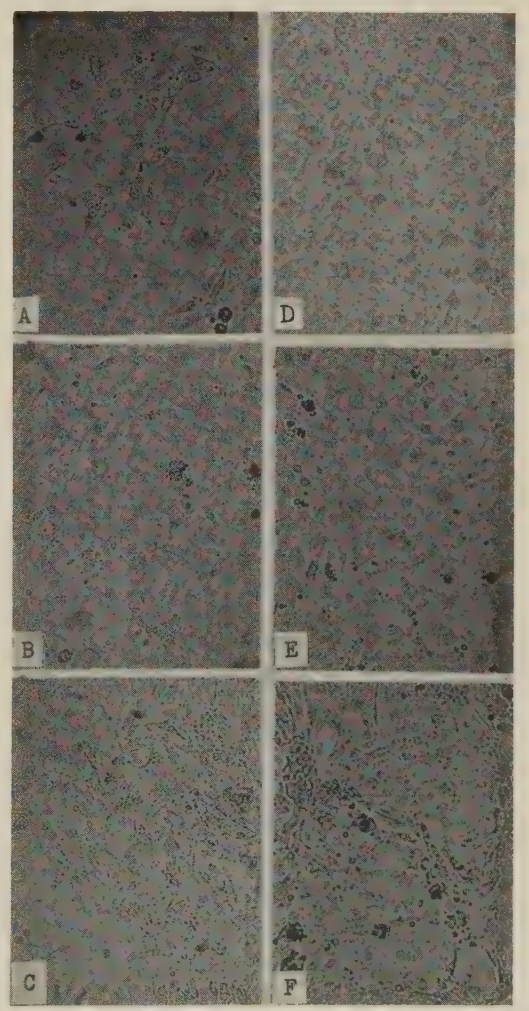

FIG. 1. Outgrowth of monkey kidney cells in SM-1 and comparison with parallel culture in lactalbumin hydrolysate-calf serum "M" medium. A-C = Outgrowth after 2½, 5, and 10 days in "M." D-F = Outgrowth after 2½, 5, and 10 days in SM-1.

The outgrowth of identical suspensions of monkey kidney cells seeded in SM-1 and in the complete medium, M, is shown in the accompanying photographs (Fig. 1). Cells seeded in SM-1 attach more slowly to the glass. The outgrowth in the first 48 hours may be inferior compared to that obtained in the serum-containing medium. By the third day, however, as indicated in the photographs, the outgrowth in SM-1 compares favorably in both amount and quality with the outgrowth in the complete medium. The two sets of cultures are comparable up to the 10th-12th day after seeding. The complete medium must be changed between the 5th and 7th days because of the decline in pH. It is not necessary to change the SM-1 medium before the 8th-10th day.

The choice of medium for replenishment is determined by the studies to be undertaken. Replenishment of cells grown in SM-1 with the M medium results in a further proliferation of the cells after a lag period of 2 days, and survival for a month or more. On the other hand, for studies uncomplicated by the addition of serum, replenishment with an amino acid mixture such as that given in Table II, in the same basic salt solution, gives a useful additional lifetime of 1-3 weeks.

The medium SM-1 was developed on the assumption that freshly isolated kidney tissue, possessing considerable endogenous reserves and biochemical capacities, would require very few organic supplements for outgrowth and survival if placed into a properly adjusted physico-chemical environment. It may be useful to summarize the steps in development. (i) Suspensions of washed monkey kidney cells in Hanks' salt solution were supplemented with single organic nitrogen sources over a range of concentrations and pH. It was found that cysteine was the only supplement in more than 50 tried which gave a significant response; cells suspended in Hanks' solution alone, or in Hanks' solution supplemented with most other nitrogen sources, disintegrated within 24 hours. (ii) The Hanks'-cysteine medium was supplemented with single nitrogen and carbon sources. Several unrelated nitrogen sources increased the extent and duration of outgrowth over that obtained with cysteine alone. On the other hand, d-ribose and several other carbon compounds improved the quality rather than the extent of outgrowth. (iii) The Hanks' cysteine medium, supplemented —rather arbitrarily—with isoleucine and d-ribose, was chosen to investigate the effects of salts and trace elements on outgrowth. The concentration of salts, particularly calcium, was found to be a critical factor for outgrowth in the absence of serum. Both ferric and cupric ions had pronounced beneficial effects. It was also found that when the pH of the medium fell below 7.2-7.3 during the first 4 days, outgrowth was inhibited; cells failed to attach to the glass and the cells already proliferating along the glass became thickened and granulated. Attachment to the glass was also retarded when the medium was more highly fortified with amino acids, phosphates, traces of certain carboxylic acids, or after prolonged metabolism. The morphological changes and lack of attachment have been found to be completely reversible. Cells held in suspensions at 37°C for several weeks, and then adjusted to pH 7.6-7.8 with bicarbonate or supplemented with calcium or zinc, were found to attach and grow into clear monolayers.

TABLE II. Amino Acid Supplement.

	mg/l
l-cysteine • HCl	30
l-isoleucine	40
l-histidine • HCl	10
l-arginine • HCl	7
l-lysine • HCl	8
l-methionine	6
l-threonine	14

Conveniently made from sterile stocks to give 40 × concentrated mixture in H_2O and diluted into salt solution with trace elements and glucose as given in Table I, with 0.9 g $NaHCO_3$ per liter. May be buffered with 8×10^{-3} M Tris at pH 7.6.

The cellular monolayers grown out in SM-1 are so firmly attached to the glass that it has not been possible to remove them by the use of trypsin or versene, or by scraping, without substantial losses. Accurate cell counts have not been obtained. As an estimation of the total number of cells recovered after outgrowth, we have relied on (i) morphological

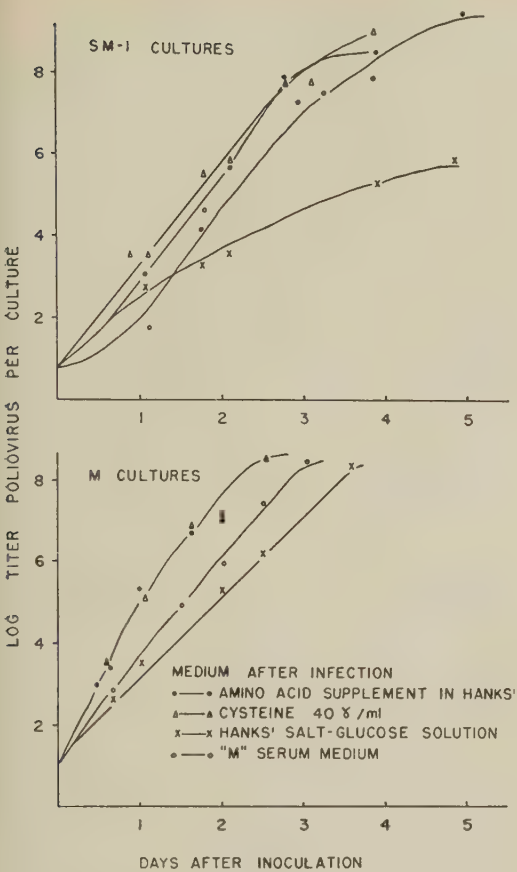

FIG. 2. Production of poliovirus in cultures grown out in SM-1 (top) and in M (bottom); and effect of medium after infection on rate of propagation. A series of 9-day-old cultures grown out in SM-1 and in M in 2 oz prescription bottles, from an inoculum of 1.7×10^6 cells/bottle, were infected with 10 plaque forming units of Type 1 poliovirus suspended in the amino acid supplement. Virus adsorbed 30 min. at 37°C. Cultures washed 3 times with PBS and in series of 6 bottles each replenished with 5 ml of medium indicated, supplemented with 8×10^{-3} M Tris pH 7.6. Arrow indicates time at which pH was readjusted to 7.4-7.6 with bicarbonate. Virus titered by plaque method, sampling from a different bottle at each point. Last point is pooled sample.

appearance of the culture and (ii) RNA and DNA recovered after a modified Schmidt-Tannhauser fractionation. The RNA and DNA recoveries indicate that after 7-10 days in SM-1, from 0.8-1.5 times the number of cells are recovered as were seeded. This compares with a recovery of 1.0-2.0 obtained in the complete medium changed at the 5th-7th day. The results obtained here for the complete medium agree with those obtained by Youngner(2) on the basis of cell count.

Virus synthesis. As a criterion of the physiological state of the cells after outgrowth in SM-1, the synthesis of poliovirus was studied. The propagation of poliovirus, Type 1, from an initial inoculum of an average of 10 plaque-forming units per culture, was followed in cultures grown out in SM-1 and in cultures of the same suspension grown out in the complete M medium. The propagation of virus throughout the two cultures, and details of the protocol, are shown in Fig. 2 and its legend. It is seen that the total yield of virus obtained from cells grown in the simple medium and inoculated and transferred to the amino acid supplement is the same as that from the control culture grown and infected in the presence of serum. The propagation of virus in the amino acid supplement is somewhat faster than in the M medium, and is more striking when the cells have been previously grown in the serum-containing medium. In this case, there may be as much as 1000 times more virus present in the cultures in the amino acid supplement at the end of 48 hours than in cultures with the complete medium. However, after further incubation of 2 or 3 days, the yield of virus from the M medium approaches that obtained in the amino acid supplement.

Since the amino acid supplement has a higher concentration of cysteine than the M medium, or other media usually used for virus cultivation, the effect of cysteine on virus propagation was studied. The data presented in Fig. 2 show that cysteine can completely replace the amino acid supplement and, even in the case of cells previously grown out in the simple medium, supports a more rapid propagation of virus than the M medium. Preliminary studies(4) indicate that the more rapid propagation of virus is due to a shorter virus growth cycle.

The amino acid supplement may be used advantageously as the nutrient in the agar overlay when assaying virus by the Dulbecco-Vogt plaque technic. The rate of appearance of plaques during assay of poliovirus Types 1, 2, and 3, on M cells overlaid with the

TABLE III. Effect of Amino Acid Supplement in Agar Overlay on Plaque Formation by Poliviruses on Monkey Kidney "M" Cells.

Exp. #*	Virus stock	Overlay medium	Plaques per bottle																				Titer (plaque forming units per ml of stock)
			Day 2							Day 3							Day 5						
1	Type 1, Brunhilde	C.S.†	1	3	2	7	3	2	5	6	3	0	18	9	10	11	7	7	8	6	5		19	13	14	12	13	12	12	10	10	7	62
		AAS†	6	6	7	9	15	12	10	5	5	13	10	9	13	14	21	14	13	10	11	18	12	11	17	16	21	14	13	11	11	18	70
2	Type 2, M.E.F.	C.S.	0	0	0	0	0	0					2	1	1	0	0	3					4	7	2	9	10	8					88
		AAS	7	11	3	5	5	4					8	15	4	11	16	5					10	15	20	14	20	17					220
3	*Idem*	C.S.	0	0	2	0	2	0	1	1	1	0	12	17	16	8	11	16	9	13	5	8	16	23	19	10	17	13	16	9	17		195
		AAS	11	14	13	20	17	14	19	8	12		28	24	17	30	29	30	27	20	23		29	28	20	34	31	32	31	22	29		390
4	"	C.S.	1	1	1	0	0	0	0	0	0	0	2	2	2	1	3	0	0	0	0	0	2	2	4	4	2	0	0	0	0	0	49
		AAS	2	3	2	2	3	3	1	3	2	1	2	6	2	5	5	3	5	3	3	1	23	10	6	10	9	7	9	4	4	6	310
5	Type 3, Saukett (Stock A)	C.S.	4	1	2	4	3	6	2	4	0	0	21	12	11	13	9	14	11	11	12	7	24	17	14	17	17	20	21	19	14	13	88
		AAS	11	16	16	16	13	15	3	11	8	4	23	26	23	22	21	20	13	19	20	13	28	29	26	27	23	27	16	22	21	20	119
6	*Idem*	C.S.	0	0	0	0	0	0	0	0	0	0	10	6	3	4	6	6	4	5	7	10	14	13	9	5	9	11	6	8	12	11	49
		AAS	12	2	3	12	4	0	6	5	5	4	22	13	6	8	14	10	7	9	13	4	23	18	11	13	20	16	13	12	19	8	78
7	Type 3, Saukett (Stock B)	C.S.											0	0	1	1	0	0					2	1	3	3	1						10
		AAS											2	1	5	6	1	6					6	9	11	10	5	7					30

* Exp. 1: .2 ml inoculum/bottle. Exp. 2, 3, 4: Repeat titrations with stock diluted 1.33×, 3×, and 7×, using .1, .2, and .2 ml inoculum/bottle, respectively. Exp. 5, 6: Repeat titrations with Stock A Saukett, .2 ml inoculum/bottle. Exp. 7: .2 ml inoculum/bottle, Stock B Saukett.

† C. S. = Calf serum in agar overlay. AAS = Amino acid supplement in agar overlay.

amino acid supplement in agar compared to the rate of appearance in the calf serum-agar overlay(5) is given by the data in Table III. Each assay was done on outgrowth cultures from kidney cells from different monkeys. The variation in final titer obtained for Types 2 and 3 indicates the variable susceptibility of the different cultures to these types. With all 3 poliovirus types, plaques appeared more rapidly in the amino acid supplement-agar overlay than in the calf serum-agar overlay. This acceleration was particularly striking with the slower growing Types 2 and 3. The final titer on Types 2 and 3 was also higher in every assay in the amino acid supplement than in the calf serum.

The susceptibility of SM-1 cells to poliovirus has been studied by the plaque technic. The data already presented in Table III indicate the rate and number of plaques expected with the different viruses on M cells with the amino acid supplement overlay. A comparative assay on SM-1 cells and M cells with this overlay would indicate any difference in the intrinsic cellular susceptibility to the virus. The SM-1 cells are sensitive to neutral red, and it is necessary to add the neutral red several days after the agar to develop the plaques. Types 1, 2, and 3 were assayed on a series of 10 bottles each of SM-1 cells and of M cells, grown in parallel from the same suspension of trypsinized kidneys. After decanting the growth fluid, the bottles were mixed so that virus inoculum was added from the same pipette in a random order to the series of 20 bottles. Neutral red was added 3 days after the agar overlay, and the plaques were read 4 hrs later. The data in Table IV show that the number of plaques developed on SM-1 cells was the same as the number developed on the M cells. This finding indicates that the cultures grown out in the simple medium have the same intrinsic susceptibility to poliovirus as the usual cultures grown out in serum. It also suggests that the difference in plaque count between M cultures in the calf serum-agar overlay and in the amino acid supplement-agar overlay is a reflection of the metabolism of the cells prevailing in these two media.

TABLE IV. Comparative Susceptibility of SM-1 and M Cultures to Poliovirus.

Virus type	Culture type	Overlay medium	No. of plaques/bottle after 3 days										Avg plaques per bottle
1	SM-1	AAS	12	13	10	11	18	14	11	16	13	27	14.5
Brunhilde	M	AAS	11	10	14	13	14	21	13	11	10	17	13.4
2	SM-1	AAS	9	9	5	11	8	13	8	14	6	7	9.0
M.E.F.	M	AAS	11	10	9	8	8	11	9	13	10	5	9.4
3	SM-1	AAS	10	6	8	4	4	1	8	7	5		5.3
Saukett	M	AAS	9	10	3	9	2	6	2	6	2	5	5.4

Discussion. The present studies indicate that suspensions of monkey kidney cells may be grown out in a simple synthetic medium in the absence of any added protein and compare favorably, in appearance and capacity to support poliovirus synthesis, with suspensions grown in the serum-containing M medium.

Under the conditions which have been studied, cysteine was found to be a specific requirement for successful outgrowth. The function of cysteine is not clear. It has not been possible so far to replace it with inorganic reducing agents, or with other organic sulfur compounds. In view of the effect of slight changes in the ionic balance of the medium during outgrowth, it is possible that these efforts were unsuccessful because the agents interfered with the necessary ionic conditions.

Outgrowth in the absence of serum was found to be sensitive to the concentration of the different salts, particularly calcium. A concentration of calcium as high as 4 times that occurring in normal serum has been incorporated into the medium. Although calcium may regulate many metabolic activities, it is considered that its beneficial effects during outgrowth may be due in part to protection of the cells during the physico-chemical adjustment to *in vitro* conditions. It may easily be observed that monkey kidney cells agglutinate at pH 4.5, suggesting a high concentration of carboxylic acid residues at their surface. If this is the case, at an alkaline pH, calcium may serve to stabilize the cells by shielding of the charged groups. It is also possible that calcium serves as a bridge through which the carboxylic acid groups are bound to the negatively charged glass. If this is a prime mechanism for the anchoring and spreading of cells along the glass surface, the pH, calcium concentration, and anionic components of the medium would be regulating factors for successful outgrowth.

Serum has heretofore been an absolute requirement for the outgrowth of monkey kidney cells. The fact that cells may be grown out and maintained for 2 to 3 weeks in the absence of serum suggests that its role is not predominantly nutritive during this period. It is possible that serum is largely protective, serving as an effective buffer to maintain the particular environment necessary for outgrowth. Biophysical studies have indicated that the elasticity of the mammalian cell membrane is probably due to tightly bound serum protein(6). It should be possible to simulate to some extent the stability afforded by bound protein by the adjustment of the physico-chemical environment. Consideration of some of the physical factors involved in stability and reversible attachment may enable one to design the medium of choice for the particular studies in tissue culture to be undertaken.

One immediate application of these findings would seem to be the production of vaccine from high titer virus obtained from monkey kidney cells grown in a medium free of added animal proteins. The binding of serum protein by animal cells means that after outgrowth in serum, even after washing, there is considerable contamination with serum protein. Although there has been little evidence of protein sensitization after inoculation of vaccines grown in monkey kidney cultures, the problem has raised a considerable controversy, particularly as to the effect of repeated injections over the years.

Summary. A synthetic medium containing only cysteine, iso-leucine, d-ribose in a salt-glucose medium, buffered with Tris (hydroxymethyl aminomethane) supports the outgrowth of monkey kidney cells. The outgrowth is comparable in both rate and amount with the lactalbumin hydrolysate-calf serum (M) medium and supports the synthesis of poliovirus. Propagation of poliovirus throughout a culture in either liquid or agar medium occurs more rapidly in the presence of a simple amino acid supplement than in the serum-containing medium. Cysteine can completely replace the amino acid supplement for propagation of Type 1 in liquid cultures.

The technical assistance of Miss Selva Reissig for the virus assays is gratefully acknowledged.

1. Rappaport, C., *Bull. World Health Organization,* 1956, v10, in press.

2. Youngner, J. S., *J. Immunol.,* 1954, v73, 392.

3. Melnick, J. L., *Ann. N. Y. Acad. Sci.,* 1955, v61, 754.

4. Rappaport, C., Howes, D., Reissig, M., and Melnick, J. L., unpublished data.

5. Hsiung, G. D., and Melnick, J. L., *Virology,* 1955, v1, 533.

6. See Danielli, J. F., for discussion in Bourne, G., *Cytology and Cell Physiology,* 1948 Ed., Clarendon Press.

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Effects of 2-Ethylamino-1,3,4 Thiadiazole HCl on Uric Acid Production in Man.* (22295)

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(Introduced by Joseph H. Burchenal.)

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A compound having the formula, 2-ethylamino-1, 3, 4 thiadiazole HCl, was found by Oleson, *et al.*(1) to have antitumor activity in various laboratory animals. Further testing by Clarke(2) confirmed the activity against sarcoma 180 and Burchenal and Dagg (3) demonstrated a definite prolongation of the survival time of mice with transplanted leukemias 82 and 8174. Because of its antitumor effect in animals, Farber(4) initiated therapeutic trials in humans. He has supplied us with preliminary clinical dosage data.

Materials and methods. Clinical evaluation by our group was started in patients with far-advanced cancer. This report deals with the observations made in the 8 adult patients treated to date. The thiadiazole was given once daily by mouth, in doses of 1-4 mg/kilo/day. In most cases therapy was continued until the development of oral toxicity. Frequent physical examinations, appropriate roentgenograms, and hematological and biochemical determinations were performed before, during and after treatment. In 5 of the 8 patients herein reported daily serum and urine uric acid determinations were performed. The method employed for the determination of uric acid was that of Archibald as described in detail by Forsham, *et al.*(5). For the urine determinations this was modified as follows: Duplicate specimens were set up. The first specimen was treated in the usual manner. To the second specimen, after dilution 0.01 ml Worthington liquid uricase† was added to 5 ml diluted urine in a Coleman, Jr. cuvette and the specimen was incubated at 37°C for 90 minutes. After incubation the procedure was completed as with the first specimen. The color remaining in the second specimen represented non-uric acid chromogens and was subtracted from the total. All

* This study was supported in part by grants from National Cancer Institute of the National Institutes of Health, Public Health Service; American Cancer Society; Damon Runyon Memorial Fund for Medical Research; and Lasker Foundation.

† Worthington Biochemical Co., Freehold, N. J.

TABLE I. Clinical and Uric Acid Data of Patients Treated with 2-Ethylamino-1,3,4 Thiadiazole HCl.

Age	Sex	Diagnosis	Daily dose, mg/kg	Days	Serum U.A. (mg %) Pre Rx	Serum U.A. (mg %) During Rx	Excess urine U.A., mg
25	♂	Melano CA	2	9	—	—	—
			4	3	—	—	—
38	♂	LSA	2	7	6.3	17.6	—
32	♀	Melanoma	2	7	—	—	—
			4	4			
			6	4	—	8.9	—
			4	11	1.9	6.0	—
62	♂	CA tongue	4	6	5.9	19.3	2160
			4	5	6.4	24.6	4270
20	♀	Salivary CA	4	3	3.9	13.8	1272
			4	4	4.5	13.2	1400
40	♀	CA breast	4	7	4.6	15.0	4176
			4*	3	4.8	12.9	2509
			4*	5	4.1	12.1	4951
			4*	6	3.6	11.6	7216
64	♂	CA kidney	4	4	5.9	13.2	4879
39	♀	CA cervix	4	5	6.7	11.2	1469

* Given with equal doses of nicotinamide.

urines were analyzed in this manner. In addition occasional blood specimens were subjected to the uricase test to determine the specificity of those values reported as uric acid. In no instance was any significant amount of non-uric acid chromogen found in the blood.

Results. No evidence of tumor regression so far has been detected in any of the patients treated. Toxic effects have been limited to severe glossitis which occurred regularly in 4-6 days at a dose of 4 mg/kg/day. Smaller doses were tolerated for longer periods of time but similar toxicity occurred when approximately the same total dosage had been achieved. The clinical and dosage data are given in detail in Table I.

An interesting and as yet unexplained biochemical abnormality that occurred regularly was the observation that coincident with administration of the drug there was a prompt and marked elevation of serum uric acid which promptly returned to normal on cessation of administration of the agent. In order to attempt to elucidate this phenomenon, studies of urinary uric acid excretion were done in 5 of the patients. With each course of therapy there was found to be a rise in urinary uric acid excretion occurring promptly and paralleling the rise in serum uric acid. Table I indicates for each course of therapy the excess urine uric acid as compared with the average urine uric acid for a similar control period.

Fig. 1 illustrates the observations made in

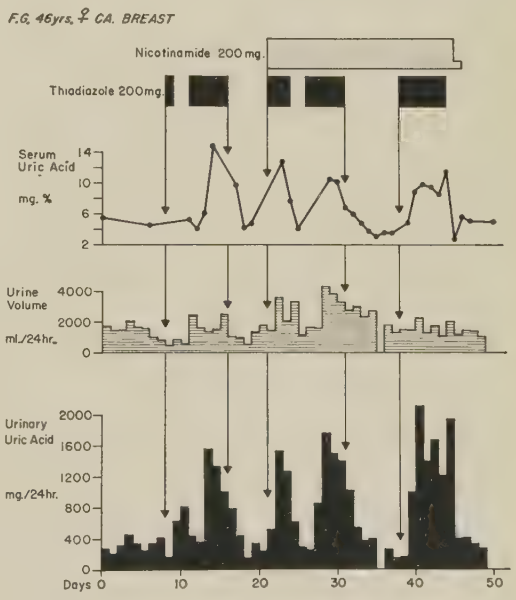

FIG. 1. Serum and urine uric acid in a patient treated with 2-ethylamino-1,3,4 thiadiazole HCl and nicotinamide.

one of the patients to whom this agent was administered. This patient, a 46-year-old woman with carcinoma of the breast and diffuse osseous metastases received 4 separate courses of medication. The dosage was 200 mg daily for 3 to 6 days in each course. On each occasion there was a rise in serum uric acid from normal levels of less than 6 mg% to levels of 10.6 to 15.0 mg%. Simultaneously the urine uric acid rose from control levels averaging 350 mg per day to levels of 1500 to 2300 mg per day. With each course severe glossitis occurred and required cessation of treatment. There was no definite evidence of regression of tumor tissue in this patient during or after treatment. Her blood urea nitrogen remained unchanged throughout this period of observation.

Inasmuch as Oleson(1) demonstrated and Clarke(2) confirmed that nicotinamide was capable of reversing the anti-tumor effect of thiadiazole, it was thought of interest to evaluate the effect of nicotinamide upon the uric acid abnormality produced by thiadiazole. Simultaneous administration of 200 mg daily of each agent produced a rise in serum and urine uric acid which was similar to that produced by thiadiazole alone. Nicotinamide did not prevent the development of oral toxicity.

Discussion. The mechanism of the uric acid abnormality occurring with administration of 2-ethylamino-1, 3, 4 thiadiazole HCl is not understood. The fact that urinary and serum uric acid rose in parallel would indicate that renal mechanisms are not involved. The absence of any indication of tumor regression in these patients renders unlikely but does not completely rule out the possibility that the uric acid was a product of degradation of the nucleic acid of tumor tissue. In the case illustrated an excess of approximately 7200 mg of uric acid above the pre- and post-treatment levels was found in the urine during and immediately after a single course of treatment. Since the average content of nucleic acid in tissue is 1% and since 20% of nucleic acid is composed of purines, calculation of the amount of tissue from which this 7200 mg of uric acid could derive reveals a figure of 3600 g. It might be expected that lysis of this amount of tumor or other tissue would be detectable. A third possibility is that there is a direct increase in uric acid synthesis, occurring as a result of the administration of this compound. This possibility has been neither proven nor disproven. Further studies are under way to investigate this problem and to attempt to correlate the rise in uric acid with nitrogen balance.

Summary. Oral administration of 2-ethylamino-1, 3, 4 thiadiazole HCl to patients with advanced cancer has been shown to cause a marked rise in serum uric acid levels and in total urinary uric acid excretion.

1. Oleson, J. J., Sloboda, A., Troy, W. P., Halliday, S. L., Landes, M. J., Angier, R. B., Semb, J., Cyr, K., Williams, J. H., *J. Am. Chem. Soc.,* 1955, v77, 6713.

2. Clarke, D., personal communication.

3. Burchenal, J. H., and Dagg, M., personal communication.

4. Farber, S., personal communication.

5. Forsham, P. H., Thorn, G. W., Prunty, F. T. G., Hills, A. G., *J. Clin. Endocrinol.,* 1948, v8, 15.

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The Structure of Nuclear Membrane in Larval Gonads of *Heliothis obsoleta*.*†‡ (22296)

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Evidence from electron microscope studies suggests that the nuclear membrane, at least in part, is not a continuous structure but is interrupted at intervals by pores, nodes, granules or ring-like formations. For example, the nuclear membrane of the amphibian egg is a double-layered structure with a continuous inner layer and a porous outer layer(1). The nuclear membrane of *Amoeba proteus* likewise is a 2-layered structure, but here the porous layer constitutes the innermost and the continuous layer the outermost lamella of the membrane(2,3). Other cells showing some form of porous structure in the nuclear membrane are oocytes of the salamander(4), kidney tubule cells(5), acinar cells of the pancreas(6), nerve cells from spinal ganglia (7,8), insect salivary gland cells(9-11), echinoderm eggs(12), several different types of rat cells(13), and chick embryonic cells (14).

Since the evidence for the porous nature of the nuclear membrane is based upon relatively few studies and since, if true, it would have important bearing on our notion of the mode of transfer of materials through it, further studies related to this subject seem warranted.

Materials and methods. Pieces of gonads from the larval insect *Heliothis obsoleta* (*Lepidoptera*, corn ear worm) were fixed for 30 minutes in buffered osmic acid at pH 7.25 (15), embedded in methacrylate(16) and sectioned at 0.025 μ by means of an International Rotary Minot type microtome(17) equipped with a glass knife(18). In Fig. 1, parts (1), (2), (3), and (4) were made by an RCA model EMU-2B electron microscope; part (5) was made with an RCA model EMU-3B electron microscope.

Results. The appearance and distribution of the nuclear membrane pores, or annuli, are clearly displayed in Fig. 1. The pores are best observed in oblique sections where they can be seen to be evenly distributed throughout the nuclear membrane at an average distance apart of approximately 1300 A (Fig. 1-1, 1-2, 1-5). In Fig. 1-2 the section is through a folded membrane showing bits of it (A) cut obliquely and the remaining part cut more or less longitudinally through the long axes of the pores.

The pores have an average diameter of about 280 A. They are encompassed by a relatively dense portion of the nuclear membrane measuring approximately 320 A in thickness (Fig. 1-3 and 1-5). Thus, the total diameter of the pore and surrounding membrane is approximately 920 A. In some preparations the relatively dense layer lining the pores appears smooth (Fig. 1-1, 1-2, and 1-3), while in others a somewhat granular appearance is observed (Fig. 1-5). The small dark granular mass appearing in the center of some of the pores (Fig. 1-5) may possibly represent material that was in the process of diffusing through them at the time the tissue was fixed.

Sections cut through the long axes of the pores show the profiles of the membranes constituting the walls to be darkly outlined (Fig. 1-2 and 1-4). In Fig. 1-4 the nuclear membrane is displayed as a single layer with an estimated thickness of from 60 to 70 A.

Discussion. From the literature cited in this paper it becomes evident that in many types of cells the nuclear membrane contains porous structures. Their orderly arrangement within the membrane and the fact that they have been demonstrated independently

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† Work performed in part under the auspices of the U. S. Atomic Energy Commission.

‡ Thanks are due Dr. T. C. Evans for permission to use the electron microscope for part of this study.

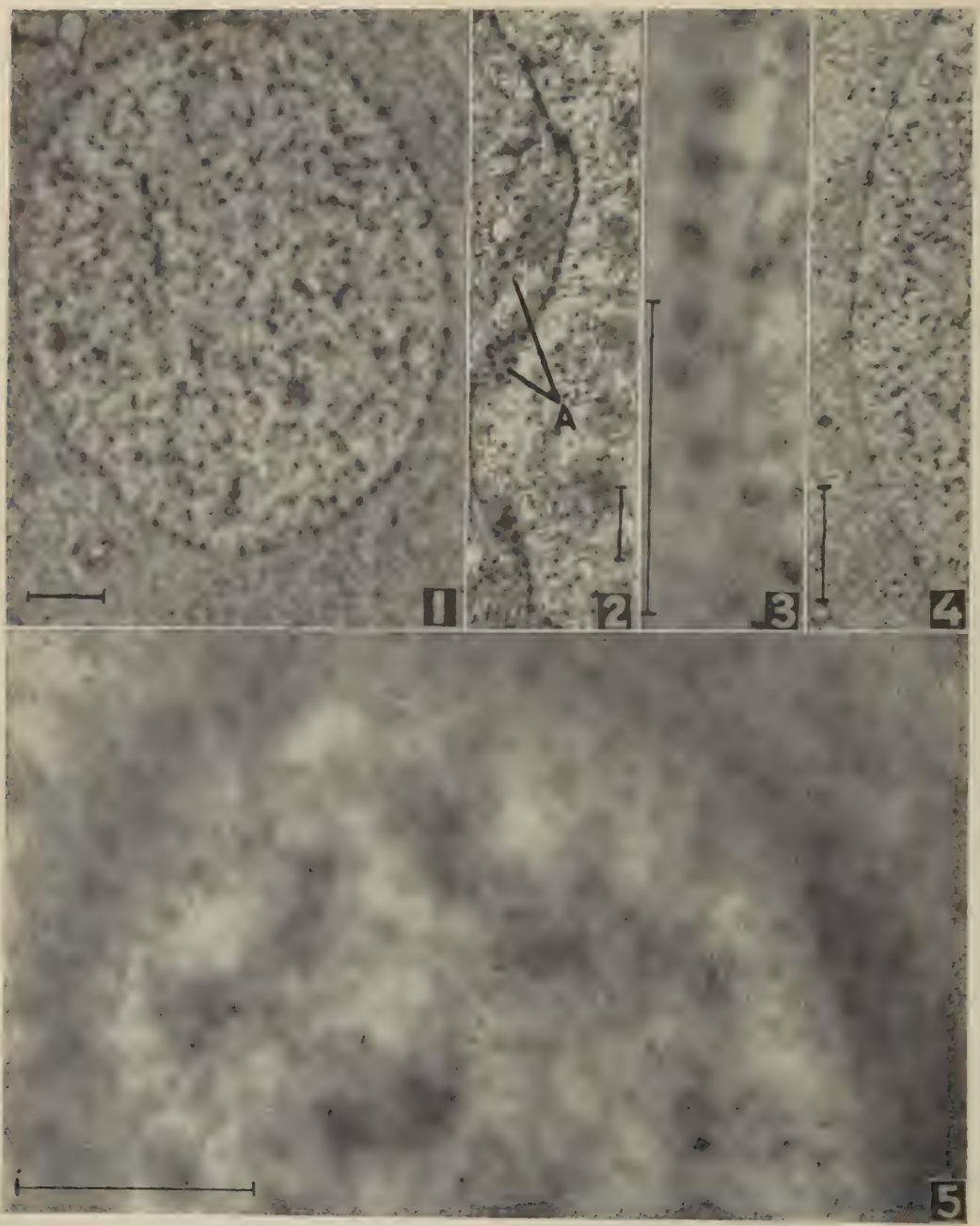

FIG. 1. Section through nuclear membrane showing pores cut either obliquely (1), (2A), (3) and (5), or longitudinally (2) and (4). The scale represents 1 μ.

by several different investigators is evidence against the view that they are artifacts. It has been suggested that they function as passageways for the diffusion of large molecules through the membrane(6).

It has been demonstrated that in certain cells bits of the nuclear membrane may be pinched off into the cytoplasm where they may contribute to the formation of the endoplasmic reticulum(10,11). In other cells, the

outer layer of the membrane is thought to be continuous with the endoplasmic reticulum; in such cases, it has been calculated that about one-tenth of the nuclear surface is in direct contact with the cytoplasmic matrix through pores, and the remaining surface is in indirect contact with it through the enclosed cavities of the endoplasmic reticulum (13). Such a close relationship between the nuclear membrane and endoplasmic reticulum is not apparent in the germ cells of the larva *Heliothis obsoleta.*

Summary. 1. Electron microscope studies on thin sections of germ cells of the larva *Heliothis obsoleta* have revealed the presence in the nuclear membrane of pore-like structures which are spaced about 1300 A apart. The pores are approximately 280 A in diameter and are surrounded by a relatively dense wall about 320 A in diameter. Thus, the diameter of the pore plus the surrounding wall is approximately 920 A. 2. The pores appear to be of a size that would permit the passage of large molecules through the nuclear membrane.

1. Callan, H. G., and Tomlin, S. G., *Proc. Roy. Soc.* (London), Ser. B, 1950, v137, 367.

2. Bairati, A., and Lehmann, F. E., *Experientia,* 1952, v8, 60.

3. Harris, P., and James, T. W., *ibid.,* 1952, v8, 384.

4. Gall, J. F., *Exp. Cell Research,* 1954, v7, 197.

5. Rhodin, J., private publication, 1954, Aktiebolaget Godvil, Stockholm.

6. Watson, M. L., *Biochim. et Biophys. Acta,* 1954, v15, 475.

7. Dawson, I. M., Hossack, J., and Wyburn, G. M., *Proc. Roy. Soc.,* (London), Ser. B, 1955, v144, 132.

8. Palay, S. L., and Palade, G. E., *J. Biophys. Biochem. Cytol.,* 1955, v1, 69.

9. Bahr, G. F., and Beermann, W., *Exp. Cell Research,* 1954, v6, 519.

10. Gay, H., *Anat. Rec.,* 1954, v120, 728.

11. ———, *Proc. Nat. Acad. Sci.,* U. S., 1955, v41, 370.

12. Afzelius, B. A., *Exp. Cell Research,* 1955, v8, 147.

13. Watson, M. L., *J. Biophys. Biochem. Cytol.,* 1955, v1, 197.

14. Kautz, J., and DeMarch, Q. B., *Exp. Cell Research,* 1955, v8, 394.

15. Palade, G. E., *J. Exp. Med.,* 1952, v95, 285.

16. Newman, S. B., Borysko, E., and Swerdlow, M., *Science,* 1949, v110, 66.

17. Geren, B. B., and McCulloch, D., *Exp. Cell Research,* 1951, v2, 97.

18. Latta, H., and Hartmann, F. J., PROC. SOC. EXP. BIOL. AND MED., 1950, v74, 436.

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Ineffectiveness of Sulfonylureas in Alloxan Diabetic Rats.* (22297)

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In explanation of the mechanism whereby the sulfonamide derivative, 1-butyl-3-p-aminobenzenesulfonylurea (BZ55) produces hypoglycemia in animal and man, Franke and Fuchs(1), Bertram, Bendfeldt and Otto(2) and Achelis and Hardebeck(3) postulate that the sulfonylurea interferes with the production of glucagon. In support of this hypothesis, these investigators refer to studies by others who demonstrated that some sulfonamide derivatives may reduce the blood sugar of alloxan diabetic animals(4,5). Further, Achelis and Hardebeck state that the sulfonylurea used by Franke and Fuchs(1), and by Bertram, *et al.*(2), produces a decrease in the blood sugar of some alloxan diabetic rabbits though they do state their impression that there may be no decrease in the blood sugar of severely diabetic alloxanized rats. In view of the paucity of data concerning this question, it became pertinent to determine whether or not the sulfonylureas are hypoglycemic in the alloxanized diabetic rat.

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Method. Male rats, weighing from 150 to 250 g were given a subcutaneous injection of 180 mg alloxan per kilogram as a 5% solution. Forty-eight hours thereafter, those animals which developed glycosuria were maintained on 1 unit protamine-zinc insulin† per day. Approximately 1 month later, the insulin was discontinued for 2 days, and after an overnight fast, 15 rats which exhibited a significant glycosuria were selected for study. A group of 15 normal male rats weighing from 150 to 250 g were used as controls after an overnight fast. Both groups of rats were given a gavage of 100 mg 1-butyl-3-p-tolylsulfonylurea‡ per kilogram body weight as a 2% solution in 0.5% sodium bicarbonate, adjusted to pH 8.0. Immediately before and at hourly intervals for 5 hours after the gavage, blood samples were taken from the clipped tails into 0.1 ml pipettes. The blood sugar concentration was determined by the Nelson procedure(6). The blood sugar concentration for each interval after the gavage was expressed as the per cent of the pregavage level for each animal.

Results. The mean ± SE response of the blood sugar concentration to the ingestion of tolylsulfonylurea by the normal and alloxan diabetic groups of rats is illustrated in Fig. 1. The mean ± SE initial blood sugar concentration was 83.0 ± 3.0 mg in the normal group and 352 ± 17.5 mg% in the alloxan diabetic group. Whereas the normal rats developed a statistically highly significant diminution in the blood sugar level within 1 hour after the ingestion of the sulfonylurea which persisted for four hours, no significant change in the blood sugar occurred in the diabetic rats.

Discussion. The administration of tolylsulfonylurea to fasted alloxan diabetic rats does not produce any significant change in the blood sugar whereas normal rats exhibit a marked hypoglycemia under similar conditions. Since the administration of alloxan results in necrosis of the beta cells of the Islets of Langerhans and a consequent cessation of insulin production, the hypoglycemic response to the ingestion of tolylsulfonylurea must be related to the availability of insulin. Further, the absence of any hypoglycemic response in the diabetic rats suggests that the hypoglycemic response of the normal rat cannot be due to an inhibition of glucagon production or utilization. Likewise, the hypoglycemic response of the intact animal cannot be due to some acute non-specific hepatotoxic action of the sulfonylurea since such action would be present in the alloxanized as well as the normal animal.

The above indicates that the response of normal animals is due to an increase in the availability of insulin. Such an increase may result from either an increased production of insulin consequent to direct stimulation of the Islets of Langerhans or to a decreased destruction of insulin consequent to an inhibition of the enzyme which catalyzes the destruction of insulin (insulinase), or to both factors. That the inhibition of insulinase plays a role in the response to the sulfonylurea by normal rats is revealed in the observation that the tolylsulfonylurea is a noncompetitive inhibitor of insulinase *in vitro* and *in vivo*(7).

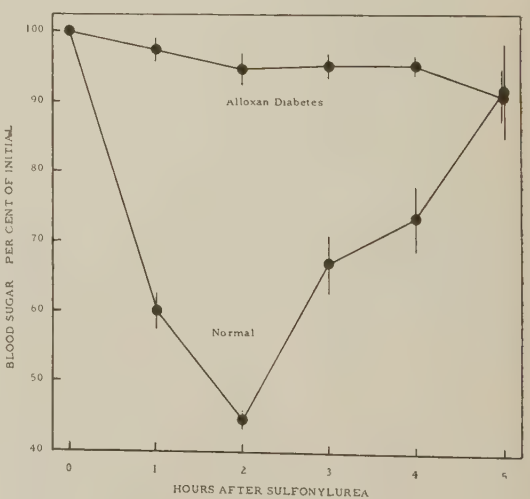

FIG. 1. Effect of tolylsulfonylurea on blood sugar of normal and alloxan diabetic rats. Blood sugar concentration at each hourly interval is expressed as % (mean ± SE) of initial concentration.

† We are indebted to the Eli Lilly Co. for generous supplies of insulin.

‡ We are indebted to Dr. C. J. O'Donovan of the Upjohn Co. for generous supplies of 1-3-butyl-p-tolylsulfonylurea ("Orinase").

Summary. Oral administration of 100 mg 1-3-butyl-p-tolylsulfonylurea per kg body weight to normal rats results in a marked hypoglycemia within one hour which persists for 4 hours. No significant change occurs in blood sugar of similarly treated alloxan diabetic rats.

1. Franke, H., and Fuchs, J., *Deutsche Med. Wochnschn.*, 1955, v80, 1449.

2. Bertram, F., Benfeldt, E., and Otto, H., *ibid*, 1955, v80, 1455.

3. Achelis, J. D., and Hardebeck, K., *ibid.*, 1955, v80, 1455.

4. Holt, C. v., Holt, L. v., Kroner, B., and Kuhnau, J., *Arch. exp. Path. Berlin*, 1955, v224, 66.

5. ———, *Die Naturwissenschaften*, 1954, v41, 166.

6. Nelson, N., *J. Biol. Chem.*, 1944, v153, 375.

7. Mirsky, I. A., Perisutti, G., and Diengott, D, *Metabolism*, 1956, v5, 156.

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Effect of CO_2 on Blood Lactic Acid in Cats.* (22298)

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Fenn and Asano(1) have observed in cats an increased acidity or fall in the alkali reserve of the blood during CO_2 inhalation, which could not be explained by movements of sodium, potassium, or chloride between tissues and plasma, and Shaw and Messer(2) have similarly observed a fall in the bicarbonate level during CO_2 breathing.

These experiments were undertaken to help clarify these findings and to identify if possible the acid mobilized by CO_2.

Procedure. The effect of high CO_2 tension on blood lactic acid was investigated in cats. Each animal was anesthetized by intraperitoneal injection of dial with urethane. Urethane has been found to raise the blood sugar, but not the lactic acid and allows for a large and rapid effect of adrenalin on the blood lactic acid(3). Four different gas mixtures were used, 10%, 20%, 30%, and 40% CO_2 in oxygen. Thus in every case the experimental animal was assured of a sufficient supply of oxygen to avoid any possibility of anoxic lactic acid formation. The desired gas mixture was inhaled by the cat from a spirometer through respiratory valves and a tracheal cannula. Blood samples were taken from the right carotid artery and were immediately deproteinized with trichloroacetic acid to stop glycolysis. After the tracheal and arterial cannulation, heparin was injected (10 mg/kg of body weight). One sample was always taken just before the onset of CO_2 inhalation. The lactic acid was determined colorimetrically by the method described by Barker and Summerson(4).

Results. The findings of these experiments are illustrated in the accompanying graphs. Increments of lactic acid in m. eq./liter above the rather variable initial blood level are plotted against time after the beginning of CO_2 inhalation. To save space, a logarithmic time scale has been used. In the graphs on the left, the different concentrations of CO_2 were inhaled for 10 minutes, (solid lines) after which the cat breathed air, (dotted lines) while in those of the right, the CO_2 was inhaled for one hour or more and a few curves are continued as dotted lines during recovery breathing air. The initial levels of lactic acid corresponding to each curve are indicated by points with appropriate symbols plotted just to the right of each graph.

With 10% CO_2 there is often a slight fall, but never a definite increase in lactic acid. The increase with 30% and 40% CO_2 is on the average somewhat greater than with 20%. The cats were unable to take 40% CO_2 for

* This work was supported in part by the Ernest L. Woodward Fund of the University of Rochester.

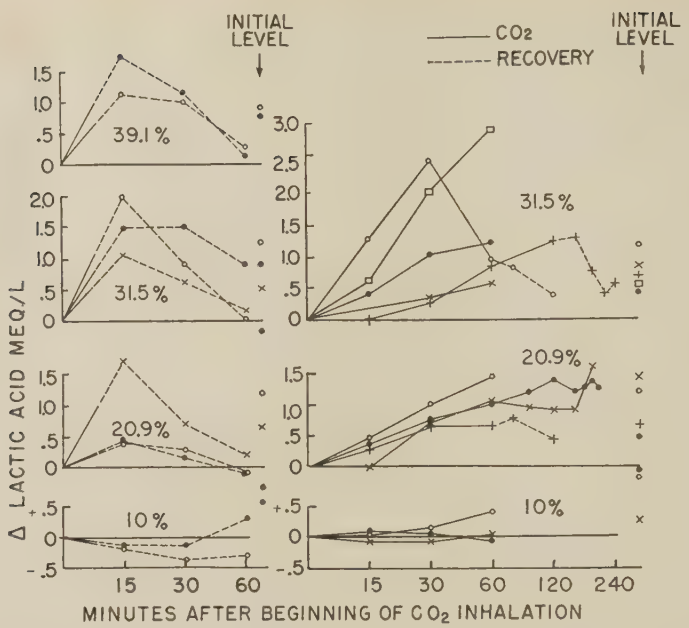

FIG. 1. Changes in lactic acid concentration of blood resulting from inhalation of different concentrations of CO_2, as indicated on the graph. Abscissae represent time after beginning of CO_2 inhalation in logarithmic units. Duration of CO_2 period was uniformly 10 min. in the graphs on the left; in graphs on the right the duration is longer as indicated by length of solid line. Broken lines represent recovery periods breathing air. The initial level of lactic acid before CO_2 is indicated by the appropriate symbol for each experiment on the right of the graph.

periods much longer than 10 minutes without cessation or serious depression of respiration and the possibility of anoxia. With 10 minute exposures to CO_2 the lactic acid reached a maximum at 15 minutes and then returned to normal in about one hour. With longer exposures the lactic acid continued to rise as long as the CO_2 was breathed or at least for one hour. In 3 out of 4 cases where recovery was followed after a prolonged CO_2 exposure, there was a slight additional rise of lactic acid when the CO_2 was discontinued. A similar "off effect" may account for the fact that the 15 minute values in short term exposures (taken after 5 minutes of recovery) seem to be on the average higher than the 15 minute values during the prolonged breathing of CO_2. No correlation was found between the initial level of lactic acid and the magnitude of the increase under similar CO_2 concentrations and no other single factor seems to account for the wide variation in response that occurs in different cats with identical CO_2 mixtures. It is likely that a combination of experimental factors and individual differences is involved.

Discussion. The release of adrenalin by CO_2 inhalation in cats has been observed by the resulting contraction of a previously denervated nictitating membrane by Fenn and Asano(1). It is assumed that the rise in blood lactate in these experiments is at least partially due to the action of the liberated adrenalin(5).

The rise in lactic acid found in these experiments after inhalation of 30% CO_2 for 60 minutes was 0.5 to 2.9 m. eq. per liter. Fenn and Asano(1) under comparable conditions found a decrease in bicarbonate (measured at constant pCO_2) of 4.16 m. eq. per liter. The lactic acid therefore is only partially sufficient to explain the acidity previously observed. In the experiments of Shaw and Messer(2) it was reported that inhalation of 10-11% CO_2 for 40 minutes caused a decrease of bicarbonate (at constant pCO_2) of 1.9 m. eq. per liter (4.2 vol %). This figure is in good agreement with the results of the present ex-

periments although there seems to be some discrepancy in the threshold CO_2 concentration required for lactic acid formation. In the experiments reported here 10% CO_2 never caused a rise in lactic acid concentration, but this is close to the threshold and a deeper anesthesia or a slightly larger respiratory dead space in the experiments of Shaw and Messer might have increased the alveolar CO_2 sufficiently to cause an effect.

In contrast to the results of these experiments, high CO_2 tensions have been reported to cause a fall in lactate in dogs(6), as well as an increased breakdown of phosphocreatine in isolated muscle(7). Further an increase in lactate has been reported during a respiratory alkalosis in dogs(6) and in a heart-lung preparation(8), and also following the injection of an alkaline solution in dogs(9,10). This suggests a metabolic buffering mechanism whereby more lactic acid is produced when the pH rises, and less lactic acid when the pH falls. In consideration of such data, the results of the present experiments, showing an increase in lactic acid on inhalation of CO_2, require some explanation, possibly on the basis of a species difference. The data of Giebisch, Berger, and Pitts(6) indicate a fall of approximately 1.0 mMol of lactate per liter in dogs that had been breathing 20% CO_2-80% O_2 for 75 minutes, while in the experiments on cats presented in this paper, there is never any evidence of a drop in lactate at any time up to nearly 3 hours with 20%, 30%, or 40% CO_2 in the inspired air.

This apparent contradiction in the results with cats and dogs may be explainable by a greater dominance or influence of the sympathetic nerves in cats(11). It is possible that the metabolic buffering mechanism seen in dogs tends to occur also in cats during CO_2 inhalation, but is masked by a release of adrenalin, which enhances the breakdown of muscle glycogen to lactic acid. If there is any purposefulness to be discerned in the reaction observed in cats, it must depend upon some features of the response other than the change in pH since lactic acid enhances the acidity caused by CO_2. Guinea pigs apparently behave like cats in this respect since Schaefer, King, Mego, and Williams(12) have reported an increase of 5.3 m. eq./liter of lactic acid 1 hour after breathing 30% CO_2 in O_2.

Summary. Inhalation of 20-30% CO_2 in oxygen by cats causes a progressive increase in the lactic acid concentration of the blood, due in part to the liberation of adrenalin.

1. Fenn, W. O., and Asano, T., *Am. J. Physiol.,* in press.

2. Shaw, L. A., and Messer, A. C., *ibid.,* 1932, v100, 122.

3. Goldblatt, M. W., *J. Physiol.,* 1933, v78, 96.

4. Barker, S. B., and Summerson, W. H., *J. Biol. Chem.,* 1941, v138, 535.

5. Griffith, F. R., Lockwood, J. E., and Emery, F. E., *Am. J. Physiol.,* 1939, v127, 415.

6. Giebisch, G., Berger, L., and Pitts, R. F., *J. Clin. Invest.,* 1955, v34, 231.

7. Lipmann, F., and Meyerhof, O., *Biochem. Z.,* 1930, v227, 84.

8. Anrep, G. V., and Cannan, R. K., *J. Physiol.,* 1923, v58, 244.

9. MacLeod, J. J. R., and Hoover, D. H., *Am. J. Physiol.,* 1917, v42, 460.

10. Haldi, J., *ibid.,* 1933, v106, 134.

11. Hodes, R., *ibid.,* 1939, v126, 171.

12. Schaefer, K. E., King, C. T. G., Mego, J. L., and Williams, E. E., *Am. J. Physiol.,* 1955, v183, 53.

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Improved Method for Determination of Inorganic Sulfate in Biologic Fluids.* (22299)

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(Introduced by P. K. Bondy.)

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Numerous adequate technics for the determination of fairly large amounts of inorganic sulfate in biologic material have been available for many years(1-3). However, the low concentration of inorganic sulfate in the plasma (2-3 mg % as $SO_4^=$) has rendered most of these methods inadequate for use with relatively small amounts of plasma (*i.e.* one to 2 cc). The basic technic which has appeared most applicable to the analysis of small quantities of inorganic sulfate in biologic fluids is benzidine precipitation of the sulfate(4). Benzidine, a weak organic base, forms stable salts with strong mineral acids. The sulfate is insoluble in the presence of excess benzidine in acid solution and particularly in the presence of organic solvents such as acetone and alcohol(4). The optimum conditions necessary for the precipitation of benzidine sulfate have been extensively studied(4-6). The benzidine sulfate must be quantitatively determined either titrametrically(7) or colorimetrically(8). In the former, the benzidine sulfate is titrated with NaOH by the following reaction: $C_{12}H_8(NH_2)_2\,H_2SO_4 + 2NaOH = C_{12}H_8(NH_2)_2 + Na_2SO_4 + 2H_2O$. According to Peters (4) the free benzidine is so weak a base that it is not alkaline to phenolphthalin or phenolred. The H_2SO_4 combined with it may be titrated as the free acid. This procedure, although accurate, requires titrating at 100°C. Letonoff and Reinhold(9) made a notable advance in the colorimetric determination of benzidine by using sodium β naphthoquinone-4-sulfonate as a color reagent. With benzidine in alkaline solution this compound develops a red brown color which changes to red on addition of acetone. However, the spectral characteristics and stability of the final color were not adequately studied. The precipitate of benzidine sulfate, once formed, must be separated and washed free of excess benzidine. As emphasized recently by Dodson and Spencer(6), considerable losses of the precipitate may occur during these steps. There is a tendency for the precipitate to break away during decantation regardless of the type of centrifuge tube used; this they prevented by adding small amounts of powdered glass as a rough surface on which the precipitate could be packed down. The benzidine sulfate has usually been precipitated from an acetone or ethanol solution and subsequently washed with either of these solvents. In the present study it was found that small but consistent losses of the benzidine sulfate precipitate occurred when washed with either acetone or ethanol. This could be prevented by washing with a mixture of ⅔ ethanol and ⅓ ethyl ether. Benzidine sulfate is considerably more insoluble in ethyl ether than in either acetone or alcohol.

By utilizing powdered glass(6), washing the precipitate with an ethanol-ether mixture and employing the color reagent, sodium β naphthoquinone-4-sulfonate(9) an improved technic for the measurement of small amounts of inorganic sulfate (10 or more μg) was obtained.

Materials and methods. Reagents. 1. H_2O, redistilled; 2. Ethanol, 95%, redistilled over NaOH pellets; 3. Benzidine, (Fisher Scientific Co., B-259), 1 g in 100 ml redistilled ethanol, preserved in dark bottle; 4. Trichloroacetic acid, 20%, redistilled with 208 mg $BaCL_2$ in 2 ml H_2O/200 g trichloroacetic acid (analytical reagent); 5. Sodium borate, (Fisher Scientific Co. S-248), 1 g in 100 ml of 0.1 *N* NaOH, stored in pyrex bottle. 6. Sodium sulfate, anhydrous, A. R., 1.4788 g/l of water to give a 100 mg % $SO_4^=$ solution. 7. Benzidine hydrochloride, purified according to Letonoff and Reinhold(9), 0.1606 g

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benzidine hydrochloride are placed in 200 ml volumetric flask, dissolved in about 50 ml water at 50°C, cooled to room temperature, diluted to mark and stored in the cold; 8. Sodium 1, 2-naphthoquinone-4-sulfonate (S. N.S.) (Eastman-Kodak 1372), purified: 2 g of crude compound are dissolved in minimum amount of boiling water, filtered through heated funnel, salted out with absolute ethanol, by adding ethanol drop wise to filtrate, filtered by suction on Büchner funnel, washed with absolute ethanol, dried with ether and stored in dark bottle. The solution must be freshly prepared each time by dissolving 150 mg in 100 ml water;† 9. Ether, A.R.; 10. Acetone, A.R.; 11. Powdered glass, washed, (Fisher Scientific Co. S-248) 15 ml heavy wall centrifuge tubes are used. All glassware should be rinsed with dilute nitric acid.

Procedure. All urine and serum samples are diluted to give a concentration of 20-140 μg $SO_4^=$ per cc. To 2 ml of the diluted sample are added 2 ml of water and 4 ml of 20% trichloroacetic acid. Samples are mixed, allowed to stand for 10 minutes and centrifuged for 10 minutes. From the supernatant fluid 2 ml aliquots are withdrawn and introduced into centrifuge tubes. To each of these tubes 5 ml of 1% benzidine solution is added. The mixtures are stirred and stirring rods washed with small amounts of ethanol. Centrifuge tubes are covered with parafilm and placed in ice-bath overnight. On following day‡ 3 to 5 mg of glass powder is added to each tube and precipitated benzidine sulfate is separated by centrifugation at 2250 rpm for 15 minutes. The supernatant fluid is decanted, with care taken not to loosen any of the precipitate. The tubes are drained, inverted on filter paper, 5 to 10 minutes and mouths of tubes are then wiped with tissue paper. The outsides of tubes are washed with acetone by medicine dropper. After draining for 1 minute, the insides of the inverted tubes are rinsed with ethanol and tubes are drained for 5 minutes. The mouths of tubes are again wiped with tissue paper and tubes are placed upright. Ten ml of an alcohol-ether mixture (1:2) is added to each tube and the precipitate suspended by a stirring rod. Care is taken to break the precipitate completely in order to dissolve all excess benzidine. The glass rods are washed with the same alcohol-ether mixture and tubes are centrifuged at moderately high speeds for 15 minutes. The supernatant fluid is decanted and tubes drained for 5 minutes. The tubes are then wiped and washed inside and outside as before. One ml of sodium borate solution is then added, the precipitate is resuspended and the tubes placed into a water-bath with occasionally stirring at about 60°C until the precipitate completely dissolves. To each tube 10 ml of water and 1 ml of the color reagent (sodium 1:2 naphthoquinone-4-sulfonate) are added and allowed to stand for 5 to 6 minutes. Two ml of acetone are then added to each tube and the solutions are mixed, centrifuged and supernatant fluid is poured into colorimeter tubes. Care is exercised that identical numbers of minutes elapse between addition of color reagent and of the acetone to each tube. The optical density of the solutions is determined in the Coleman Junior Colorimeter at 490 mμ. All analyses were done in triplicate or quadruplicate.

Standards. Stock standard benzidine hydrochloride is diluted 30 fold. To 7 centrifuge tubes 1 to 7 ml of this diluted standard solution is added and the volumes made up to 10 ml with water. A blank tube containing 10 ml of water is prepared with each determination. To all tubes sodium borate, color reagent and acetone are added as outlined before.

Results. Characteristics of benzidine-sodium β naphthoquinone-4-sulfonate (S.N.S.) complex. Fig. 1 illustrates the absorption spectrum of final color compound. Maximum optical density occurs at 490 mμ. It is

† In the present study, in contrast to Letonoff (9), a definite fall in optical density of standards was noted if fresh reagent was not prepared with each set of analyses. However, as the optical density of the standards and unknowns are lowered by proportional amount no error is introduced by using S.N.S. previously prepared and stored in the cold (4°C).

‡ Complete precipitation will occur within 3 hours; leaving samples overnight is a matter of convenience.

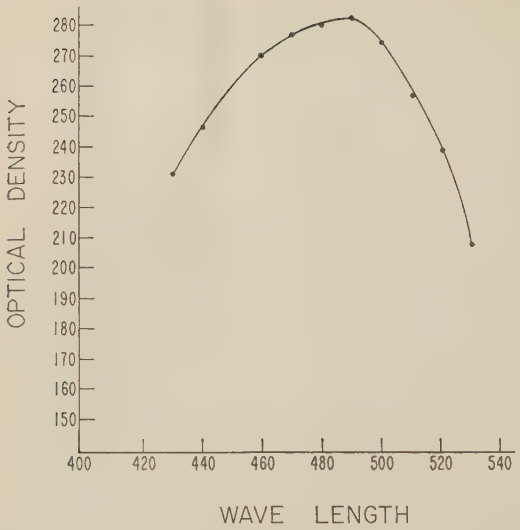

FIG. 1. Absorption spectrum for final benzidine-β naphthoquinone-4-sulfonate compound as determined on Coleman Junior Spectrophotometer.

of interest that this wave length also represents the optimum length for the color developed by interaction of this reagent and amino acids(10), the amine groups of benzidine combining with the S.N.S. to give the observed color. At this wave length, the standard curve follows Beer's law quite closely between zero and 50 μg of sulfate (the benzidine hydrochloride equivalent to these amounts of $SO_4^=$). Rarely at 50 μg, and invariably above this level the curve deviates from a straight line as illustrated in Fig. 2. Therefore for maximum accuracy the precipitate of benzidine sulfate should be equivalent to 50 γ of sulfate or less. Although the slope of this curve is readily reproducible small variations in optical density of the standards from one analysis to the next necessitates that a set of standards be run simultaneously with the unknowns. Maximum color development occurs in 5 to 6 minutes and this is stable for at least 2 hours if room temperature is relatively constant. After 2 hours optical density decreases slowly and proportionally in all samples.

Recovery of benzidine sulfate from standard solutions, urine and serum. In Table I are listed recoveries of sulfate from solutions of known concentration. The mean recovery for this series was 98.8% (range 94-104) however, recoveries below 97% were seen only with the sample containing 10 γ of sulfate. Sulfate added to serum and urine was quantitatively recovered (Table II). The mean recovery from serum was 100.5% (range 97-102) and from urine 99.0% (range 97-104). Duplicate determinations on fresh

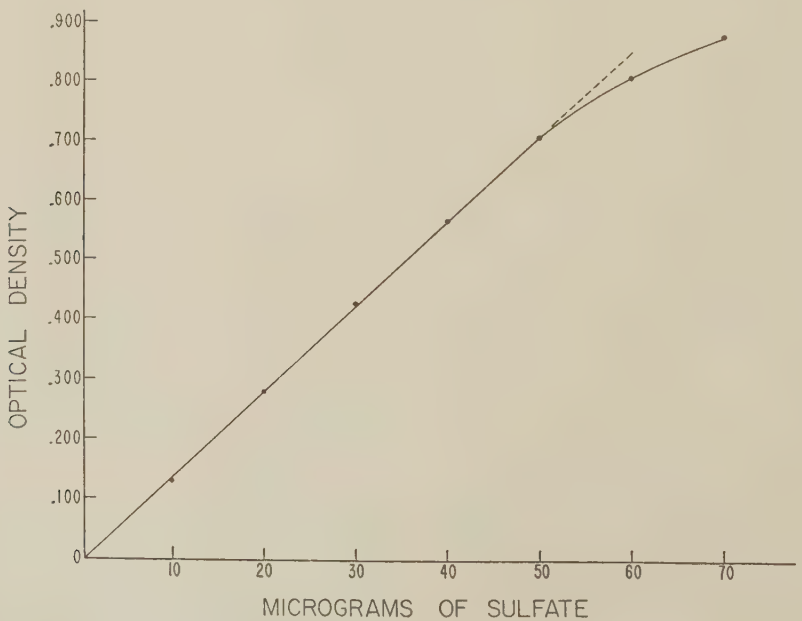

FIG. 2. Calibration curve for benzidine-β naphthoquinone-4-sulfonate compound at 490 mμ.

TABLE I. Analysis of Pure Sulfate Solutions. Five determinations* in each solution.

Sulfate,† μg	Found—Mean	Found—Range	% variation
10	10.1	9.4-10.4	94-104
20	19.7	19.4-20.0	97-100
30	30.1	29.0-30.4	98-103
40	39.7	39.0-40.0	99-100
50	49.9	49.2-52.0	98-104
60	59.5	58.0-62.0	97-103
70	69.5	68.0-70.7	97-101

* Each determination done in triplicate.
† Sulfate present in initial aliquot of solution.

or frozen serum and urine were reproducible within a range of ± 2.5%. If, however, serum or urine were stored at 4-5°C in the unfrozen state for 24 hours or longer an increase in concentration of inorganic sulfate was almost always noted, probably as a consequence of some degeneration of organic sulfates in these biologic fluids.

The mean concentration of inorganic sulfate in the sera of 15 normal subjects was 2.84 mg % (range 2.23-3.90). These values are comparable to those obtained by other investigators using benzidine or other technic (1,3,11).

It has been reported that inorganic phosphates in concentrations found in human urine are coprecipitated as benzidine salts and must be removed prior to precipitating inorganic sulfate(5,7). With the present technic, however, urinary inorganic phosphates in concentrations up to 150 mg % did not interfere with sulfate precipitation. This was investigated by determining urinary sulfates before and after precipitation of phosphate as magnesium ammonium phosphate(12).§

The reproducibility and accuracy of the present method was equal to or better than other procedures utilizing larger quantities of plasma or serum(1,2,3,7,13). However, it must be stressed that, *like* all procedures requiring quantitative recovery of a small precipitate, utmost care is essential.

Summary. 1) An improved method for the determination of inorganic sulfate in biologic fluid has been presented.

2) The results compare favorably with other methods requiring larger quantities of serum or plasma.

TABLE II. Recovery of Sulfate Added to Serum and Urine.

Serum	Added sulfate, μg	Total—Expected, μg	Total—Found, μg	% of expected $SO_4^=$ recovered
μ				
1 (15.4)*	15.0	30.4	31.0	102
"	30.0	45.4	46.4	102
"	30.0	45.4	44.2	97
2 (30.0)	15.0	45.0	46.0	102
"	15.0	45.0	44.0	98
"	30.0	60.0	60.2	100
3 (41.6)	3.3	44.9	45.0	101
"	6.6	48.2	49.4	102
Urine				
1 (11.2)*	30.0	41.2	39.8	97
2 (22.5)	20.0	42.5	41.5	98
"	15.0	37.5	37.3	100
3 (21.6)	22.5	44.1	44.8	102
"	15.0	36.6	38.0	104
4 (16.0)	22.5	38.5	38.0	99
5 (32.0)	22.5	54.5	55.7	102
"	15.0	47.0	47.6	101
6 (9.6)	7.5	17.1	16.7	98

* Sulfate present in initial aliquot of serum and urine.

§ Powdered calcium hydroxide(4) was found unsuitable as an agent to precipitate phosphate because varying amounts of sulfate were found to be coprecipitated with calcium phosphate.

1. Kahn, M., and Postmontier, R. S., *J. Lab. and Clin. Med.*, 1925, v10, 317.

2. Loeb, R. F., and Benedict, E. M., *J. Clin. Invest.*, 1927, v4, 33.

3. Denis, W., and Reed, L., *J. Biol. Chem.*, 1926-27, v71, 191.

4. Peters, J. P., and Van Slyke, D. D., *Quantitative Clinical Chemistry Methods*, 1932, Baltimore, Williams and Wilkins Co.

5. Schwartz, B. M., Thesis for degree of Doctor of Medicine, Yale University, 1939.

6. Dodgson, K. S., and Spencer, B., *Biochem. J.*, 1953, v55, 436.

7. Cope, C. L., *ibid.*, 1931, v25, 1183.

8. Wakefield, E. G., *J. Biol. Chem.*, 1929, v81, 713.

9. Letonoff, T. V., and Reinhold, J. G., *ibid.*, 1936, v114, 147.

10. Frame, E. G., Russell, J. A., and Wilhelmi, A. E., *ibid.*, 1943, v149, 255.

11. Hoffman, W. S., and Cardon, R., *ibid.*, 1935, v109, 717.

12. Fiske, C. H., *ibid.*, 1921, v47, 59.

13. Cuthbertson, D. P., and Tompsett, S. L., *Biochem. J.*, 1931, v25, 1937.

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Comparison of Pharmacological Effects of Lysine and Arginine Vasopressins.* (22300)

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The neurohypophyseal octapeptides, oxytocin and vasopressin, have been separated from the posterior lobes of oxen as highly purified substances by du Vigneaud and his collaborators(1,2). Related studies of hog pituitary glands led to the isolation of an oxytocin identical in all respects with the oxytocin of the ox(1). Hog vasopressin, however, differs from ox vasopressin in one respect: the arginine of ox vasopressin is replaced by lysine(3). The latter hormones are therefore conveniently designated as arginine or lysine vasopressin according to the amino acid characteristic of each. The pharmacological effects of the two vasopressins differ in one interesting respect which is described in this report.

Material and methods. Two preparations containing lysine vasopressin of different degrees of purity were used: one, a natural lysine vasopressin, purified but not regarded as the pure hormone (No. 686) and the other, an extract of acetone-desiccated hog posterior lobes (No. 700). The purified natural lysine vasopressin was prepared in the Department of Biochemistry of Cornell University Medical College; we are indebted to Prof. V. du Vigneaud for the gift of this preparation. The extract of hog posterior lobe powder was made by suspending the powder in dilute acetic acid at pH 3.0, boiling for 1 minute and filtering after cooling. The solutions of this extract and of the purified natural lysine vasopressin, also dissolved in aqueous acetic acid solution at pH 3.0, were sealed in ampoules and immersed in boiling water for 10 minutes before final storage in the refrigerator at 1.0°C. The vasopressor potency of the stored solutions in terms of U.S.P. standard were as follows: No. 686, 1.34 units per ml, and No. 700, 3.54 units per ml. The absolute potency of the samples at the time of use were 134 units per mg for No. 686, and 1.35 units extracted from 1 mg of powder for No. 700. The methods of assay have already been described(4). They employ intravenous injections in the anesthetized rat (vasopressor response) and lactating rabbit (milk-ejecting response) and in the trained unanesthetized hydrated dog (antidiuretic response). The antidiuretic response was also determined after subcutaneous injection in the hydrated unanesthetized rat by the method of Burn (5). Lastly, the avian depressor response to intravenous injection was measured in the anesthetized fowl by Coon's method(6).

Results. Because the vasopressor assay was considered the most convenient and accurate to perform in terms of expenditure of time, the potency as found by this method was chosen as the standard of comparison with the other pharmacological properties. The pattern of the pressor response to lysine vasopressin with regard to dose-response relationship and duration of action was identical with that elicited by the U.S.P. reference standard or by arginine vasopressin. The variability in potency from ampoule to ampoule is indicated by the following result: 134.3 units per mg with a standard error of $\pm$ 5.6 units for No. 686 from vasopressor assays in 12 rats with as many ampoules.

In Table I, the relative potencies of natural arginine and lysine vasopressins compared by the different tests are listed. In addition, the potencies of the extract of hog posterior pituitary powder are recorded; this extract also contained oxytocin which accounts for the high potency of this extract as tested for its effects on the blood pressure of the fowl and

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† Research Assistant supported by a research grant (H-1788) from National Heart Institute of the National Institutes of Health, Public Health Service.

TABLE I. Relative Potency of Arginine and Lysine Vasopressins.

Preparation	Tests of potency: Pressor† (rat, i.v.)	Antidiuretic (Dog, i.v.)	Antidiuretic (Rat, s.c.)	Avian depressor (fowl, i.v.)	Milk-ejecting (rabbit, i.v.)
Arginine vasopressin (natural)	100	100	100	14	17
Lysine vasopressin (natural)	100	15	110	16	22
Extract of hog post. pituitary*	100	17	88	127	130

* Contained both vasopressin and oxytocin.
† In these experiments, the pressor effects of the 3 preparations in the rat were arbitrarily assigned a value of 100.

on the pressure in the lactating mammary gland of the rabbit. The avian vasodepressor and milk-ejection effects of lysine vasopressin are qualitatively indistinguishable from those of arginine vasopressin. Also the avian depressor-pressor and milk-ejection-pressor ratios show within the limits of experimental error a satisfactory agreement. In contrast to the findings in the above mentioned bioassays, the relative antidiuretic potency of lysine vasopressin after intravenous injection in the dog is much lower than that of its arginine congener and requires more detailed consideration.

Whenever highly purified lysine vasopressin is administered intravenously to the unanesthetized hydrated dog, the antidiuretic response differs qualitatively as well as quantitatively from that observed following the injection of arginine vasopressin. First, the urine flow in the first 5-minute period following the intravenous injection of lysine vasopressin is about the same as in the second 5-minute period, whereas the antidiuresis produced by the arginine compound is commonly much more marked in the second period (Fig. 1). Secondly, lysine vasopressin as assayed intravenously for its antidiuretic action in the dog, has about one-sixth of the potency of arginine vasopressin when both are compared with their vasopressor potencies in the rat (Table I).

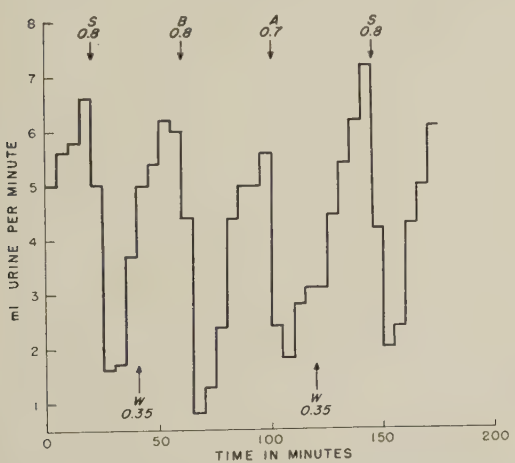

FIG. 1. Antidiuretic response in trained unanesthetized dog after intrav. injections of: S. U.S.P. Posterior Pituitary Reference Standard, 1 mU/ml. B. Extract of human infundibular stem, No. 722 (containing arginine vasopressin), 1:20. A. Purified lysine vasopressin, No. 686, 6.7 mU/ml according to vasopressor assay in the rat. W represents fractions of liters of water by stomach tube in addition to 0.4 liter at −75 and −60 min.

In an endeavor to compare the antidiuretic potencies of the vasopressins by a method employing a different route of administration, assays were performed in the rat by the method of Burn. Eleven groups of 4 animals each were employed using 2 dose levels of each preparation in at least 10 groups (40 rats). The parameter of time to 50% excretion of the water load was chosen since it appeared to be less influenced by the retention of urine in the bladder. Under these conditions, lysine vasopressin, like its arginine relative, evokes an antidiuresis which is equivalent to its pressor potency. To learn whether there is a general difference in the response of the dog and rat to lysine vasopressin, quantitative estimates of the vasopressor potency of the hormone after intravenous injection were made in the dog. The vasopressor action of lysine vasopressin was identical in the dog and rat in terms of U.S.P. standard.

Discussion. Experiments with highly purified arginine vasopressin demonstrated that the antidiuretic hormone is probably identical

with the vasopressor hormone of the neurohypophysis of oxen. In the quantitative determination of arginine vasopressin in an extract either type of assay could be employed. With the isolation of another naturally-occurring vasopressin which has a 1:6 murine vasopressor-canine antidiuretic ratio of doses, assays for antidiuretic hormone must take into account the source of the preparation, the species used for assay and the route of administration. The low potency of lysine vasopressin as an antidiuretic agent in the dog could be attributed to a difference in the response of another species. This explanation is untenable as a general statement because the vasopressor potency of lysine vasopressin, in comparison with the U.S.P. standard, is the same in the rat and dog.

Other investigations, some of which are still in progress, indicate that extracts of the neurohypophysis of man, the monkey, dog, rat, ox, sheep and camel, when assayed by intravenous methods, show a 1:1 ratio for vasopressor and antidiuretic potencies. The work described in this paper suggests that arginine vasopressin is the principal if not the only neurohypophyseal hormone responsible for these actions in the above mentioned species.

Summary. 1. The pharmacological effects of lysine and arginine vasopressins are the same with respect to their relative vasopressor, milk-ejecting and avian depressor potencies. 2. The ratio of pressor potency to antidiuretic potency is 1 for arginine vasopressin and 6 for lysine vasopressin when the antidiuretic potency is determined by intravenous injection in the hydrated, unanesthetized dog. On the other hand, this ratio of potencies is 1 for both vasopressins when the antidiuretic potency is determined by subcutaneous injection in the hydrated, unanesthetized rat. 3. Lysine vasopressin, compared with the U.S.P. standard or arginine vasopressin, is equally potent as an intravenous pressor agent in the dog and rat. Potency refers to *relative* potency; potency in units per mg is much higher for purified arginine vasopressin. 4. Lysine vasopressin is peculiar to the hog. The vasopressin of man, the macaque monkey, dog, rat, ox, sheep and camel appears, by pharmacological criteria, to be arginine vasopressin. These conclusions are tentative and must be confirmed by the isolation of the hormone from each species, other than the ox and hog, together with the demonstration of the amino acid composition of each vasopressin.

1. Pierce, J. G., Gordon, S., and du Vigneaud, V., *J. Biol. Chem.*, 1952, v199, 929.

2. Turner, R. A., Pierce, J. G., and du Vigneaud, V., *ibid.*, 1951, v191, 21.

3. Popenoe, E. A., Lawler, H. C., and du Vigneaud, V., *J. Am. Chem. Soc.*, 1952, v74, 3713.

4. van Dyke, H. B., Adamsons, K., Jr., and Engel, S. L., *Rec. Prog. Horm. Res.*, 1955, v11, 1.

5. Burn, J. H., Finney, D. J., and Goodwin, L. G., *Biological Standardization*, Oxford, 1950, 187.

6. Coon, J. M., *Arch. internat. Pharmacodyn.*, 1939, v62, 79.

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Arterial Pressures in Tourniquet Shock. (22301)

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In this study arterial pressure was recorded during the entire period of tourniquet shock in the rat. The object was to correlate the pressure with the course and fatal outcome of the shock state.

Method. Our standard method for the production of shock in rats involves a high unilateral tourniquet applied to the left hind extremity for 5 hours. The technic was described previously(1). White male rats weighing about 250 g were used. Prior to removal of the tourniquet, the animals were anesthetized with 2% Nembutal, given intraperitoneally, and each received 0.2 cc liquid

heparin by vein. The right femoral artery was then cannulated (the carotid artery was used in a few instances) and connected to a mercury manometer. Practically all parts of the apparatus were treated with Monocote (Armour) to provide a non-wettable surface. Control readings were taken for several minutes before the tourniquet was released. Direct arterial pressures were obtained throughout the course of shock in 15 animals. In an occasional rat there was need for an extra dose of anesthesia during the period of observation.

Results. The arterial pressure curves were basically similar in all 15 rats with tourniquet shock. Fig. 1 illustrates 3 such curves in animals which died at 1½, 3, and 5 hours respectively after removal of the tourniquet. 1½ and 5 hour animals represented the shortest and longest survivals in the whole group. The mean survival time was 2 hours and 40 minutes.

The initial pressure in the various animals ranged from 104 to 130 mm, and there was no significant change during the control period. Almost immediately after tourniquet release, *i.e.* within a minute or so, there was an abrupt fall in pressure to levels of about 70 to 80 mm. This was probably due to reactive hyperemia in the injured limb as indicated by a bright pink flushing of the paw promptly after the tourniquet was removed. However, participation of nervous factors or possibly toxic material from the damaged extremity is difficult to exclude with certainty.

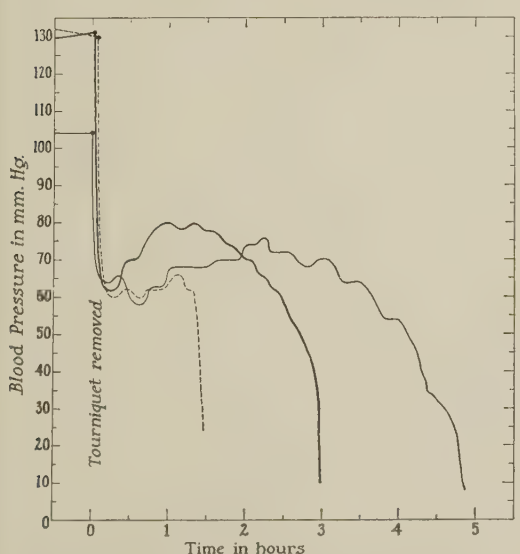

FIG. 1. Arterial pressures in tourniquet shock.

After the initial sharp drop, the arterial pressure showed a further mild decline for a short interval of about 10 to 15 minutes. Then over approximately the next one to 4 hours, depending on the duration of life, and continuing until shortly before death, there was sustained hypotension. During this time, which comprised most of the shock state, the pressure was maintained within a relatively narrow range of about 60 to 70 mm or 70 to 80 mm. That this occurred in spite of progressive shock with marked fluid loss in the injured limb amounting to about 5% of body weight(1) and reduction in effective circulating blood volume to about half the normal value(2), was presumably due to compensatory adjustments of the circulation such as vasoconstriction, increased cardiac action, etc. In fact the compensatory mechanism was probably responsible for the tendency of arterial pressure to rise slightly in some animals during the latter part of the shock state.

Terminally, over a period of a few to about 20 minutes, there was a collapse of the arterial pressure which was associated with death. This was the only interval during the entire course of shock when the pressure fell below 40 mm Hg. The most abrupt decline occurred in animals with the shortest survival time (Fig. 1).

The reason for the terminal drop in pressure was obscure. There was no clear indication that it resulted from impairment of cardiac action. No dilatation of the heart or pulmonary edema was found at autopsy. In fact examination failed to reveal any significant lesions beyond those observed in animals sacrificed prior to the terminal phase. It is unlikely, and at least not proven, that the cause was abrupt capillary stasis, either generalized or local. Through reduced blood flow and progressive anoxia in tourniquet shock, there is profound tissue injury with impairment of vital functions but exactly how this relates to sudden decline in arterial pressure and death is not clear.

Discussion. We are unaware of previous studies of arterial pressure curves in rats with tourniquet shock. Pressures generally resembling those in this study have been noted in dogs with tourniquet(3,4) or compression shock(5) and also in dogs with plasmapheresis(6).

The arterial pressures following release of a unilateral 5 hour tourniquet are of interest in connection with the problem of irreversibility. We have shown that this fulminant form of shock is not characterized by an irreversible stage. To the contrary it is reversible via fluid therapy, with or without replacement of the tourniquet, even up to the time of death (7,8). In addition, evidence is available that no bacterial factor is involved(9).

It might be said that this type of shock lacks irreversibility because it is not associated with drastic hypotension. Throughout most of tourniquet shock in our study the arterial pressure remained above 60 mm Hg. Blood pressure becomes especially pertinent when the tourniquet and hemorrhagic forms of shock are compared, since extremely low pressures, *i.e.* 30 to 45 mm Hg, maintained over a period of time, often form the basis of standard irreversible hemorrhagic shock(10, 11). Such technics, which involve loss of whole blood rather than plasma, presumably give more marked anoxia and tissue injury than occur in tourniquet shock and this might be a critical factor in the development of an irreversible state.

Regardless of the validity of this argument, there is no doubt that profound tissue injury can result from marked and rapid loss of plasma. In fact such injury in the tourniquet and related procedures is clearly out of proportion to the degree of hypotension. For example it was shown in dogs with compression shock that systemic blood flow was seriously impaired even when arterial pressures exceeded the 50 to 70 mm Hg range(5). In dogs with acute plasmapheresis cardiac output declined more rapidly than the blood pressure and the discrepancy was more marked with loss of plasma than with comparable hemorrhage(6). Our own view is that arterial pressure is not a reliable criterion of either the severity or outcome of tourniquet shock.

As indicated, the shock we produce by release of a unilateral 5 hour tourniquet is of acute type and, if untreated, causes death within a few to several hours. Nevertheless, proper therapy will save even moribund animals. Successful reversal of the shock state can be obtained in the last few minutes of life, when arterial pressure is declining abruptly and the levels are about 30 to 40 mm. Approximately 70% of rats recover with fluid therapy alone(8) and nearly 100% with fluid plus replacement of tourniquet(7). This indicates that in the rat tourniquet shock is basically a reversible form of shock.

Summary. Arterial pressure in rats with fatal tourniquet shock shows 3 main phases: (1) an abrupt fall immediately after tourniquet release, (2) sustained hypotension within a relatively narrow range during most of the shock state, and (3) a terminal more or less rapid decline associated with death. Only in the terminal period does profound hypotension intervene, with levels below 40 mm. Although arterial pressure is not a reliable index of the severity of tourniquet shock, the absence of drastic hypotension might help explain why this form of shock is not irreversible.

1. Koletsky, S., and Gustafson, G. E., *J. Clin. Invest.,* 1946, v25, 744.

2. Koletsky, S., unpublished data.

3. Wilson, H., and Roome, N. W., *Arch. Surg.,* 1936, v32, 334.

4. Swingle, W. W., Remington, J. W., Kleinberg, W., Drill, V. A., and Eversole, W. J., *Am. J. Physiol.,* 1942, v138, 156.

5. Green, H. D., Dworkin, R. M., Antos, R. J., and Bergeron, G. A., *ibid.,* 1944, v142, 494.

6. Price, P. B., Metcalf, W., Longmire, W. P., Hanlon, C. R., and Rizzoli, H. V., *Bull. Johns Hopkins Hosp.,* 1944, v75, 14.

7. Koletsky, S., and Gustafson, G. E., *Am. J. Physiol.,* 1954, v178, 229.

8. Koletsky, S., Klein, D. E., *ibid.,* 1955, v182, 439.

9. Koletsky, S., and Gustafson, G. E., PROC. SOC. EXP. BIOL. AND MED., 1946, v62, 293.

10. Wiggers, C. J., *Physiol. Rev.,* 1942, v22, 74.

11. Wiggers, H. C., Ingraham, R. C., and Dille, J., *Am. J. Physiol.,* 1945, v143, 126.

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Removal of Blood Ammonia by Hemodialysis.* (22302)

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In this communication we wish to report our experience with hemodialysis performed on dogs with raised blood ammonia values.

The use of portacaval shunts for portal hypertension and the use of cation exchange resins have presented problems in ammonia metabolism resulting in a considerable revival of interest in the metabolism of amino acids and ammonia in liver disease(1,2). Many patients in hepatic coma and acute liver failure have demonstrated an increase in the value for ammonia in the blood(3). Elevated blood ammonia values have been found and also induced in some patients with portacaval shunts (anastomosis of the portal vein to the vena cava)(1). It seemed of interest therefore to see whether the Kolff artificial kidney would reduce the values for blood ammonia in patients and in experimental animals.

Materials and methods. A number of dogs have been subjected to hemodialysis using a modified Kolff dialyzer. Mongrel dogs weighing from 10 to 20 kg were prepared by producing an end to side portacaval shunt. The "meat intoxication" syndrome was induced by feeding of meat and urea as described by Riddell(4). Eight dogs with an elevated blood ammonia level and "meat intoxication" manifested by lethargy, blindness, ataxia, catatonia, or convulsions were treated with the artificial kidney for 3 hours using a flow of 100 ml per minute and a dialyzing surface of about 12,000 cm^2. Blood ammonia, measured by the microdiffusion technic of Conway (5) utilizing a 10 minute diffusion time(6), was measured at the beginning of the cellophane loops used for dialysis and 2 minutes later at the end of these loops, the determinations were made intermittently during the period of dialysis for the purpose of evaluating the rapidity of removal of blood ammonia.

* This work was supported by a grant from the U.S. Public Health Service.

Results. Percent removal of blood ammonia under these circumstances has been plotted against arterial concentration. (Fig. 1). It can be seen that blood ammonia concentration has been generally lowered by 50% during one passage through the artificial kidney with a tendency toward higher percentage removal with higher blood concentrations. The total amount of blood ammonia removed was estimated by multiplying the amount removed per milliliter of blood by the volume of blood dialyzed for successive twenty minute periods throughout the dialysis. This total amount removed was found to vary in 4 dialyses from 15,000 μg to 130,000 μg. When approximately 25,000 μg of blood ammonia had been removed, ammonia became detectable in the 100 liters of bath fluid used. No ammonia was ever detected in the bath fluid at the start of dialysis. The dif-

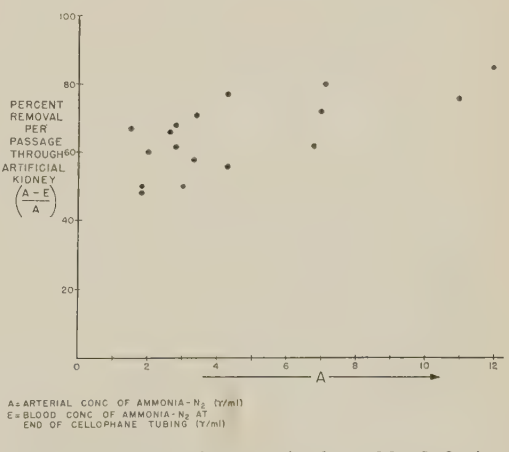

FIG. 1. Removal of ammonia from blood during one passage through a Kolff artificial kidney. Graph indicates % ammonia removed from blood of experimental animals with elevated values for blood ammonia. Note that the higher the value for blood ammonia the greater the extraction by dialysis. Normal value for blood ammonia in dogs is usually under 1 μg/ml. (Flow—100 ml/min.; dialyzing surface—approximately 12,000 cm^2.) (At a flow of 100 ml/min., clearance in ml/min. by the artificial kidney is numerically equal to percent removal.)

fusion of ammonia out of the bath fluid into the air however, may make erroneous an estimation of ammonia removal based on the amount of ammonia in the bath fluid. No fall in blood ammonia was noted on passage of blood through the artificial kidney if the cellophane loops were not immersed in the bath fluid. In fact a small but definite rise in blood ammonia was noted under these circumstances. Normal dogs without portacaval shunts when subjected to hemodialysis in the same manner as those with meat intoxication did not show any appreciable elevation of ammonia in the blood entering or leaving the kidney.

Comment. The invariable lowering of blood ammonia concentrations during passage through the artificial kidney and the appearance of ammonia in the bath water as dialysis proceeded indicated to us that hemodialysis removed blood ammonia in dogs with elevated blood ammonia levels.

We are presently using this method of removing blood ammonia to study certain aspects of liver disease. Whether hemodialysis may have any clinical value in the treatment of hepatic coma is also being investigated.

Summary. Blood ammonia, as measured by the microdiffusion technic of Conway, is removed efficiently from the blood of dogs with elevated blood ammonia levels by a Kolff type artificial kidney with a dialyzing surface of 12,000 cm^2. When flow through artificial kidney is 100 ml/minute the clearance of blood ammonia varies from about 50 ml/minute at lower arterial concentrations of blood ammonia to about 80 ml/minute at higher arterial concentrations.

1. McDermott, W. V., and Adams, R. D., *J. Clin. Invest.*, 1954, v33, 1.

2. Phillips, G. B., Schwartz, R., Gabuzda, G. J., and Davidson, C. S., *New Eng. J. Med.*, 1952, v247, 239.

3. Traeger, H. S., Gabuzda, G. J., Ballou, A. N., and Davidson, C. S., *Metabolism*, 1954, v3, 99.

4. Riddell, A. G., Kopple, P. N., McDermott, W. V., *Surgery*, 1954, v36, 675.

5. Conway, E. J., *Microdiffusion Analysis and Volumetric Error*, D. VanNostrand Co., Inc., 1947.

6. Webster, L. T., personal communication.

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Hypothalamic Stimulation of ACTH Secretion. (22303)

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It has been shown that antidiuretic and oxytocic hormones of the hypothalamic-posthypophyseal system* may stimulate the anterior lobe of the pituitary gland to discharge ACTH. It has been demonstrated that these hormones produce, when injected into normal animals, significant fall of circulating eosinophils(2), adrenal ascorbic acid (3) and cholesterol(4); these actions which have not been observed in hypophysectomized animals(3,4), are apparently due to stimulation of the adenohypophysis. Release of ACTH occurs also in hypophysectomized rats bearing a hypophyseal graft in the anterior chamber of the eye after local injection of small amount of posterior pituitary hormones into the subconjunctival space(5). This observation rules out the possible participation of a "stress" reaction in the ACTH-releasing power of posterior pituitary hormones. These results are in agreement with the hypothesis(2-6) that hormones produced in the hypothalamic nuclei may act as neurohumoral agents in the hypothalamic control of the activity of the anterior lobe of the pituitary gland.

* There is much positive evidence to support the view that "posterior pituitary hormones" are produced by cells of the nuclei supraopticus and paraventricularis and pass along the axons of the tractus supraoptico-hypophyseus into the posterior lobe which serves as a storage-release center(1).

TABLE I. Effect of Continuous Injection of Antidiuretic (Pitressin, Parke-Davis) and Oxytocic Hormones (Pitocin, Parke-Davis) on Mean Weight of Body, Hypophysis and Adrenal Glands in Normal Rats; 10 Rats in Each Series, Treated 15 Days.

Treatment	Body wt (g) Initial	Body wt (g) Final	Hypophysis wt (mg/100 g final body wt)	Adrenal wt (mg/100 g final body wt)
Pitressin (.5 U/rat)	148 ± 5	146 ± 8	4.41 ± .20	15.92 ± .8
Pitocin (.5 U/rat)	158 ± 7	154 ± 6	4.48 ± .21	15.02 ± .9
.9% NaCl	138 ± 5	145 ± 7	4.66 ± .30	11.76 ± .7
Inactivated Pitressin (.5 U/rat)	156 ± 8	162 ± 6	4.35 ± .20	10.85 ±1.1

The experiments here described confirm that both antidiuretic and oxytocic hormones may induce a conspicuous discharge of ACTH; these hormones produce, when chronically injected in normal rats, considerable modifications of adrenal weight and histology, which are prevented by hypophysectomy; injections of hormones previously inactivated with sodium thioglycollate are without any activity.

Methods. Normal and hypophysectomized male Sprague-Dawley rats, average weight 150 g, were used. All animals were given a commercial diet and drinking water *ad libitum*. Ten normal rats were given 0.5 U/rat of antidiuretic hormone (Pitressin, Parke-Davis) in daily subcutaneous injections, through 15 days; 10 rats were given 0.5 U/rat of oxytocic hormone (Pitocin, Parke-Davis) in same manner and time: 10 rats were daily treated with 0.5 U/rat of Pitressin previously inactivated in accordance with Van Dyke's thioglycollate method(7); 10 control rats were daily injected with 1 ml of 0.9% NaCl solution. An analogous experiment was carried out in rats hypophysectomized by the usual parapharyngeal approach. The rats were killed 24 hours after last injection. Pituitaries and adrenals were carefully dissected out and weighed. The pituitaries were then stained by Rasmussen-Ignesti's technic; the adrenals were stained with hematoxylin and eosin, with Sudan III and with Smith-Dietrich's method. The lipase content of adrenals was studied by Gomori's method.

Results. Body and organ weights of normal rats treated with posterior pituitary preparations are shown in Table I.

Neither antidiuretic nor oxytocic hormones cause significant changes in weights of body and anterior pituitary. After treatment with either antidiuretic or oxytocic hormones a conspicuous and significant enlargement of the adrenal glands was observed: the inactivated hormone was completely ineffective.

The histological picture of the anterior pituitary gland after treatment with active Pitressin or Pitocin showed a higher incidence of basophilic cells. Adrenal glands of animals treated with active hormones showed a characteristic enlargement of the "zona fasciculata" and a conspicuous increase of sudanophilic material (Fig. 1 and 2); lipasic activity was also enhanced. These effects were not obtained with the inactivated preparation.

In hypophysectomized rats the posthypophyseal extracts did not produce any modification of weight and of the histology of the suprarenal glands (Table II).

Discussion. The results of these experiments completely agree with Moehlig's(8) and Hantschmann's(9) observations; these authors have described adrenal hypertrophy in rabbits after treatment with whole posterior pituitary extract; Heinbecker's(10) observation that posterior pituitary hormones

TABLE II. Effect of Continuous Injection of Antidiuretic (Pitressin, Parke-Davis) and Oxytocic Hormones (Pitocin, Parke-Davis) on Mean Weight of Body and Adrenal Glands in Hypophysectomized Rats; 10 Rats in Each Series, Treated 15 Days.

Treatment	Body wt (g) Initial	Body wt (g) Final	Adrenal wt (mg/100 g final body wt)
Pitressin (.5 U/rat)	165 ± 7	155 ± 8	9.11 ± .9
Pitocin (.5 U/rat)	170 ± 9	158 ± 7	8.20 ± .7
.9% NaCl	168 ± 7	157 ± 8	9.02 ± .9

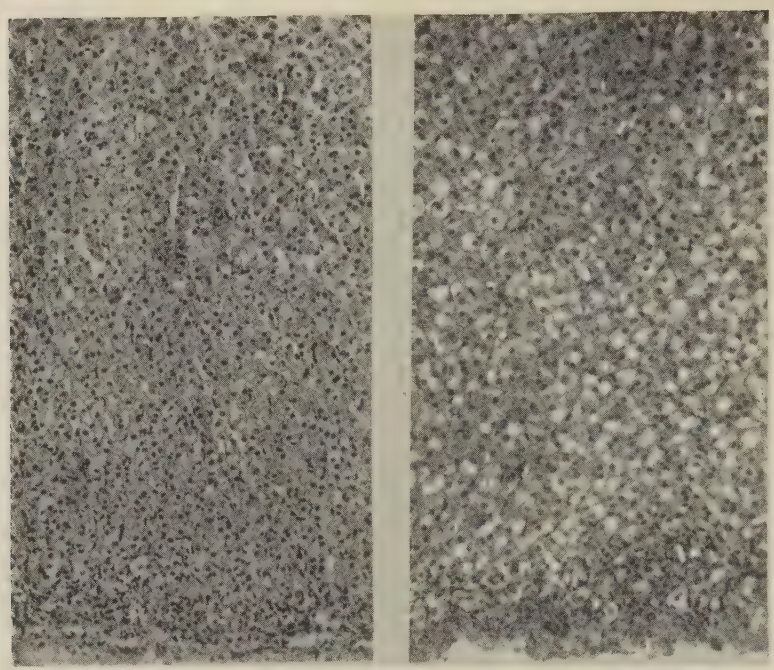

Fig. 1 Fig. 2

Adrenal cortices fixed in 10% formalin and stained with hematoxylin and eosin.

FIG. 1. Adrenal cortex of control rat.
FIG. 2. " " " rat treated with antidiuretic hormone through 15 days.

may induce a marked basophilism in the pituitary gland is also confirmed.

The evidence herein presented supports earlier observations(2-6) that both oxytocic and antidiuretic hormones can activate the adrenal cortex by causing the release of adrenocorticotrophic hormone; experiments in hypophysectomized rats eliminate the possibility that the effects observed may be due to contamination with ACTH of the injected hormones. Complete inefficacy of the inactivated posterior pituitary preparation seems to indicate that the ACTH-releasing power is strictly linked to the posterior pituitary hormonal molecule and not to an impurity contained in posterior pituitary extracts as suggested by Saffran(11).

These experiments strongly suggest that the "posterior pituitary hormones" or some related compounds may be considered as a neurohumoral agent linking the hypothalamus to the anterior pituitary through the portal blood flow(12). Considerable additional evidence is now available in favour of this hypothesis.

Green(13), Palay(14) and Scharrer(1) have found considerable Gomori-positive neurosecretory material, (containing posterior lobe hormones) in the proximity of the hypophyseal portal vessels, in the median eminence and in the infundibular stem. Rothballer(15) and Barrnett(16) have shown that after exposure of rats to pain stimuli the neurosecretory material of the hypothalamo-hypophyseal system is depleted from the median eminence and from the infundibular process into the portal vessels. Mirsky(17) has reported an acute elevation in blood antidiuretic hormone levels after various stimuli, such as pain and adrenalectomy, all associated with augmented ACTH discharge. McCann(18) observed in rats that hypothalamic lesions, which produce diabetes insipidus, uniformly block ACTH secretion; ACTH secretion appears to be produced in operated rats by Pitressin administration. Sobel(19) demonstrated that Pitressin causes an increased excretion of urinary corticoids by guinea pigs.

Summary. 1) Both antidiuretic and oxytocic hormones of the hypothalamic-posthypo-

physeal system produce, when chronically injected in normal rats, a significant enlargement of the adrenal glands and a conspicuous increase of the adrenal sudanophilic material. 2) The hormones do not produce modification in hypophysectomized animals. 3) An inactivated preparation of antidiuretic hormone is completely ineffective. 4) These results are consistent with the hypothesis that antidiuretic and oxytocic hormones of the hypothalamo-posthypophyseal system may act as neurohumoral agents linking the hypothalamus to the anterior pituitary through the portal blood flow.

1. Scharrer, E., and Scharrer, B., *Recent Progr. Horm. Res.,* 1954, v10, 183.

2. Fraja, A., and Martini, L., *Boll. Soc. It. Biol. Sper.,* 1952, v28, 407.

3. Bertelli, A., and Martini, L., *Atti Soc. Lomb. Sc. Med. Biol.,* 1952, v7, 430.

4. Martini, L., and Morpurgo, C., *Nature, Lond,* 1955, v175, 1127.

5. Martini, L., and De Poli, A., *J. Endocrin.,* 1956, in press.

6. Martini, L., *Ann. Endocr., Paris,* 1955, v16, 670.

7. Van Dyke, H. B., Chow, B. F., Greep, R. O., and Rothen, A., *J. Pharmacol.,* 1942, v74, 190.

8. Moehlig, R. C., and Osius, E. A., *Ann. Intern. Med.,* 1931, v4, 578.

9. Hantschmann, L., *Klin. Wschr.,* 1937, v16/1, 378.

10. Heinbecker, P., *Ann. Surg.,* 1946, v124, 252.

11. Saffran, M. A., Schally, A. V., and Benfey, B. G., *Endocrinology,* 1955, v57, 439.

12. Harris, G. W., *Brit. Med. J.,* 1951, v2, 627.

13. Green, J. D., *Anat. Rec.,* 1947, v99, 21.

14. Palay, S. L., *Am. J. Anat.,* 1950, v47, 338.

15. Rothballer, A. B., *Anat. Rec.,* 1953, v115, 21.

16. Barrnett, R. J., *Endocrinology,* 1954, v55, 484.

17. Mirsky, I. A., Stein, M., and Paulisch, G., *ibid.,* 1954, v55, 28.

18. McCann, S. M., and Brobeck, J. R., PROC. SOC. EXP. BIOL. AND MED., 1954, v87, 318.

19. Sobel, H., Levy, R. S., Marmorston, J., Schapiro, S., and Rosenfeld, S., *ibid.,* 1955, v89, 10.

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Actions of Reserpine and Chlorpromazine Hydrochloride on Rat Brain Oxidative Phosphorylation and Adenosinetriphosphatase.* (22304)

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The use of chlorpromazine and reserpine in treating psychiatric disorders has been discussed by several investigators(1-8). Chlorpromazine is widely used as a tranquilizing agent for mentally disturbed patients and is regarded as a general central nervous system depressant capable of potentiating certain other depressant drugs(9-11). The sedative and hypotensive actions of reserpine have been attributed to a central inhibition of the sympathetic nervous system(12-14). Although chlorpromazine and reserpine are similar in their clinical actions, a number of opposing pharmacological characteristics have been reported. In contrast to chlorpromazine, reserpine facilitates production of convulsions by caffeine or metrazol and antagonizes actions of barbiturates and anti-convulsive drugs(15-17). Chlorpromazine-HCl, at concentrations of 10 mg% (2.8×10^{-4} M) or greater, has been reported to depress oxygen uptake by brain slices(18).

In the present investigation, effects of chlorpromazine and reserpine upon two important high energy phosphate enzymatic reactions were studied. These compounds were tested separately and in combinations in order to detect possible synergistic or antagonistic actions. For comparison chlorpromazine was tested in combinations with sodium pentobarbital, the latter drug also being known to uncouple oxidative phosphorylation(19).

Materials and methods. Adult Sprague-Dawley albino rats were used. Fifteen percent brain homogenates were prepared in isotonic sucrose with a Potter-Elvehjem type

* Supported in part by grant from the Mental Health Fund of the State of Illinois.

TABLE I. Effect of Reserpine on Oxidative Phosphorylation in Rat Brain Homogenates.

Reserpine, μg/ml	△ P		△ O		P : O	
	μM	% ± S.E.	μatoms	% ± S.E.	Ratio	% ± S.E.
0	27.2	100	10.11	100	2.70	100
10	27.0	99.2 ± 1.6	10.07	99.6 ± 1.1	2.68	99.5 ± .8
25	25.5	93.4 ± 1.2†	9.70	95.9 ± 1.6*	2.63	97.6 ± 1.7
50	23.9	87.8 ± 1.8†	9.05	89.6 ± 1.8†	2.64	98.2 ± 3.1

△ P = μmoles inorganic phosphate esterified. △ O = μatoms oxygen uptake.
* $p < .05$ and † $< .01$ that differences from controls are due to chance.

homogenizer. The components added to each Warburg flask for the study of oxidative phosphorylation were as follows: in the main compartment, 100 μM of KCl, 25 μM of glycylglycine, 40 μM of K_2HPO_4, 3 μM of KOH, 0.03 μM of cytochrome c, 26 μM of Na-pyruvate, 4 μM of malic acid, 2.5 μM of K-ATP (neutralized with KOH), 16 μM of $MgCl_2$, 20 μM of NaF, and 75 mg of rat brain homogenate in a total of 1.8 ml of triple distilled water; in the sidearm, 5 mg of hexokinase (Pabst) and 50 μM of dextrose in a total of 0.2 ml; and in the center well, 0.2 ml of 10% KOH. Additions of drugs† are indicated in appropriate tables. Because of its insolubility in water, reserpine was dissolved in 10% polyethylene glycol containing 0.04 malic acid and 0.26 M Na-pyruvate, and all concentrations of the drug were added to the flasks in a total of 0.1 ml containing the substrates, so that in experiments with reserpine all flasks including controls contained 0.01 ml of polyethylene glycol as well as the components mentioned above. The vessel contents when mixed was pH 7.4. All steps directly involving the homogenate were carried out in an ice water bath until the vessels were attached to the manometers and then placed in the incubating bath. The sidearms were tipped after 6 minutes, and zero time readings were taken exactly 3 minutes after tipping each flask. Zero time flasks were removed and 0.2 ml aliquots were quickly pipetted into 2.8 ml of 0.32 M trichloracetic acid. Oxygen uptake of the experimental flasks was measured for 25 minutes at 25°C, after which 0.2 ml aliquots were also added to trichloracetic acid. Inorganic phosphate uptake was determined for each set of experimental conditions(20) and P:O ratios were calculated. The method of DuBois and Potter(21) was employed for ATPase determinations. In experiments with reserpine, the drug was dissolved in 10% polyethylene glycol containing 0.02 M malic acid, and all test amounts were added in 0.05 ml. All flasks including controls,

† Chlorpromazine hydrochloride and reserpine were kindly supplied by Smith, Kline and French Laboratories, Philadelphia, Pa., and Ciba Pharmaceutical Co., Summit, N. J., respectively.

TABLE II. Effects of Combinations of Reserpine and Chlorpromazine on Oxidative Phosphorylation in Rat Brain Homogenates.

Chlorpromazine, M × 10^4	Reserpine, μg/ml	△ P			△ O			P : O		
		μM	% inhibition by RSP ± S.E.	p*	μatoms	% inhibition by RSP ± S.E.	p*	Ratio	% inhibition by RSP ± S.E.	p*
0	0	26.3			9.50			2.77		
	50	24.2	7.9 ± 1.3	—	8.42	11.2 ± 2.0	—	2.88	−4.0 ± 1.4	—
2.5	0	23.5			8.56			2.75		
	50	18.7	20.4 ± 1.4	<.001	7.22	15.6 ± 2.7	>.1	2.60	5.2 ± 2.4	<.01
5.0	0	16.3			6.78			2.41		
	50	9.7	41.0 ± 2.0	"	4.61	31.5 ± 3.9	<.001	2.11	12.5 ± 3.5	<.01
7.5	0	6.8			4.14			1.64		
	50	2.9	57 ± 4	"	2.59	37.1 ± 3.4	"	1.10	32.3 ± 5.3	<.001

RSP = Reserpine.
* p values indicate significance of inhibition by reserpine in the presence of various concentrations of chlorpromazine as compared with reserpine inhibition in the absence of chlorpromazine.

TABLE III. Effects of Combinations of Sodium Pentobarbital and Chlorpromazine on Oxidative Phosphorylation in Rat Brain Homogenates.

Chlorpromazine, M × 10^4	Pentobarbital, M × 10^4	△ P			△ O			P : O		
		μM	% inhibition by PTB ± S.E.	p*	μatoms	% inhibition by PTB ± S.E.	p*	Ratio	% inhibition by PTB ± S.E.	p*
0	0	28.4			10.59			2.69		
	2.5	15.1	46.9 ± .9	—	5.92	44.0 ± .9	—	2.55	5.0 ± 1.5	—
2.5	0	23.6			9.32			2.53		
	2.5	12.2	48.3 ± 1.4	>.1	5.00	46.4 ± 1.0	>.1	2.44	3.5 ± 3.1	>.1
5.0	0	14.9			7.22			2.06		
	2.5	7.4	50.5 ± 1.6	>.05	3.76	47.9 ± 2.1	"	1.98	3.6 ± 5.3	"
7.5	0	6.5			4.56			1.43		
	2.5	2.9	55.3 ± 3.3	"	2.67	41.0 ± 2.9	"	1.10	23.1 ± 6.7	<.05

PTB = Pentobarbital.

* p values indicate significance of inhibition by pentobarbital in the presence of various concentrations of chlorpromazine as compared with pentobarbital inhibition in the absence of chlorpromazine.

therefore, contained 0.005 ml of polyethylene glycol and 1 μM of malic acid as well as the other necessary components. Sufficient KOH was added to the stock solution of ATP to bring the reaction mixture to pH 7.45.

Results. All of the studies represent determinations from 6 or more animals each. At flask concentrations of 25 and 50 μg/ml of reserpine, small but significant decreases in phosphate and oxygen uptake were observed (Table I), but no significant changes were found in P:O ratios(22).

The depression of oxidative phosphorylation of brain homogenate systems by chlorpromazine is shown in Tables II and III. Effective concentrations are similar to those reported by Finkelstein, Spencer, and Ridgeway(18), but are 10 times as high as those reported by Abood who used mitochondrial preparations(23). Reserpine at 8.22 x 10^{-5} M (50 μg/ml) more effectively depressed phosphate and oxygen uptake and P:O ratios in the presence of increasing amounts of chlorpromazine than in the absence of the latter drug (Table II). On the other hand, very little difference was observed in the percent depression of P, O, and P:O values by 2.5 x 10^{-4} M Na-pentobarbital in the absence and presence of increasing amounts of chlorpromazine (Table III).

Six rats were given 10 mg/kg of chlorpromazine-HCl subcutaneously and 6 control animals were given water injections. All were sacrificed after 1 hour. Animals receiving the drug were severely depressed and incontinent but were not unconscious. Oxidative phosphorylation by brain homogenates was determined in the absence and presence of Na-pentobarbital in the flasks. Small but significant increases in oxygen uptake were obtained with homogenates from animals treated with chlorpromazine in the absence and presence of 2.5 x 10^{-4} M pentobarbital in the flasks (Table IV). No changes were seen in organic phosphate uptake or P:O ratios.

Chlorpromazine up to 6.25 x 10^{-4} M resulted in little or no depression of hexokinase activity(24) so that the decrease in phosphate uptake was not due to depression of the hexokinase trapping system.

TABLE IV. *In Vivo* Chlorpromazine on Oxidative Phosphorylation in Rat Brain Homogenates.

Inj. chlorpromazine, mg/kg	Pentobarbital in flasks, M × 10^4	△ P μM ± S.E.	△ O μatoms ± S.E.	P : O Ratio ± S.E.
0	0	28.3 ± .4	10.19 ± .14	2.78 ± .03
	2.5	13.1 ± .7	5.37 ± .09	2.44 ± .11
10	0	28.5 ± .4	10.74 ± .07*	2.69 ± .04
	2.5	14.1 ± .4	5.78 ± .07*	2.44 ± .08

* p <:01 that increases over corresponding values from untreated animals are due to chance.

Reserpine up to 50 μg/ml (8.22×10^{-5} M) had no significant effect on rat brain adenosinetriphosphatase activity, and chlorpromazine at 1.2×10^{-4} M and 1.5×10^{-4} M only slightly depressed ATPase.

Discussion. Both chlorpromazine and reserpine are capable of depressing oxygen and inorganic phosphate uptake by rat brain homogenates, but at concentrations much higher than ordinary pharmacological or therapeutic doses. It is highly improbable that reserpine depresses phosphorylation *in vivo*, since single effective pharmacological doses of 1 mg/kg in animals and daily sedative doses of as little as 0.5 mg represent much lower concentrations than the 25 mg/l needed to depress phosphate and oxygen uptake in rat brain. It is interesting to note that in effective concentrations, reserpine acts synergistically with chlorpromazine in depressing phosphate and oxygen uptake and P:O ratios in brain homogenates in contrast to similar combinations of pentobarbital and chlorpromazine. Although the concentration of chlorpromazine (2.5×10^{-4} M or 89 mg/l) needed to depress oxidative phosphorylation in rat brain was much greater than the physiologically depressing dose of 10 mg/kg, small increases in oxygen uptake by brain homogenates from treated animals are suggestive that the drug may have some effect on oxidative processes *in vivo*. Small increases in rat brain oxygen uptake were reported to be insignificant by Grenell, Mendelson, and McElroy (26). However, increases in oxygen uptake have been reported by Abood(23) with mitochondria using 2×10^{-5} M (7.1 mg/l) chlorpromazine *in vitro*. This concentration is comparable to the usual pharmacological doses.

From the data presented here and in recent reports(18,19,23), it appears that mitochondrial preparations are more sensitive to certain depressant drugs than whole homogenates or slices. Studies by Johnson and Ackerman (25) showing that nuclei potentiate mitochondrial oxidative phosphorylation suggest the possibility that nuclei and perhaps other cell fractions may protect mitochondria from the actions of uncoupling drugs. Suitable studies of this subject are now being undertaken.

Summary. Oxidative phosphorylation by rat brain homogenates was depressed by chlorpromazine at 2.5×10^{-4} M or greater, while reserpine at 25 and 50 μg/ml only slightly depressed oxygen uptake and phosphorylation. Effective concentrations of reserpine appeared to depress phosphorylation synergistically with chlorpromazine. Oxygen uptake was slightly increased with brain homogenates from animals treated with 10 mg/kg or chlorpromazine. Reserpine up to 50 mg/l had no effect on enzymatic ATP breakdown, while chlorpromazine, at 1.2×10^{-4} M and 1.5×10^{-4} M depressed rat brain adenosinetriphosphatase slightly.

1. Anton-Stephen, D., *J. Ment. Sci.*, 1954, v100, 543.

2. Winkelman, N. W., *J.A.M.A.*, 1954, v155, 18.

3. Bleuler, M., and Stoll, W. A., *Ann. N. Y. Acad. Sci.*, 1955, v61, 167.

4. Kinross-Wright, V., *ibid.*, 1955, v61, 174.

5. Noce, R. H., Williams, D. B., and Rapaport, W., *J.A.M.A.*, 1954, v156, 821.

6. Kline, N. S., and Stanley, A. M., *Ann. N. Y. Acad. Sci.*, 1955, v61, 85.

7. Hollister, L. E., Krieger, G. E., Kringel, A., and Roberts, R. H., *ibid.*, 1955, v61, 92.

8. Tasher, D. C., and Chermak, M. W., *ibid.*, 1955, v61, 108.

9. Brand, E. D., Harris, T. D., Borison, H. L., and Goodman, L. S., *J. Pharmacol. and Exp. Therap.*, 1954, v110, 86.

10. Courvoisier, S., Fournel, J., Ducrot, R., Kolsky, M., and Koetschet, P., *Arch. Int. Pharmacodyn.*, 1953, v92, 305.

11. Zipf, H. F., *Arch. exp. Pathol. Pharmakol.*, 1954, v221, 76.

12. Plummer, A. J., Earl, A., Schneider, J. A., Trapold, J., and Barrett, W., *Ann. N. Y. Acad. Sci.*, 1954, v59, 8.

13. Winsor, T., *ibid.*, 1954, v59, 61.

14. Bein, H. J., *ibid.*, 1955, v61, 4.

15. Schneider, J. A., PROC. SOC. EXP. BIOL. AND MED., 1954, v87, 614.

16. Chen, G., and Ensor, C. R., *ibid.*, 1954, v87, 602.

17. Chen, G., Ensor, C. R., and Bohner, B., *ibid.*, 1954, v86, 507.

18. Finkelstein, M., Spencer, W. A., and Ridgeway, E. R., *ibid.*, 1954, v87, 343.

19. Brody, T. M., and Bain, J. A., *J. Pharmacol.*

and Exp. Therap., 1954, v110, 148.

20. Lowry, O. M., and Lopez, J. A., *J. Biol. Chem.,* 1946, v162, 421.

21. DuBois, K. P., and Potter, V. R., *ibid.,* 1943, v150, 185.

22. Youden, W. J., *Statistical Methods for Chemists,* John Wiley & Sons Inc., 1951, p24.

23. Abood, L. G., PROC. SOC. EXP. BIOL. AND MED., 1955, v88, 688.

24. Berger, L., Slein, M. W., Colowick, S. P., and Cori, C. F., *J. Gen. Physiol.,* 1946, v29, 379.

25. Johnson, R. B., and Ackerman, W. W., *J. Biol. Chem.,* 1953, v200, 263.

26. Grenell, R. G., Mendelson, J., and McElroy, W. D., *Arch. Neurol. and Psychiat.,* 1955, v73, 347.

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Transmethylation Reactions *in vivo* and *in vitro* in the Young Calf.* (22305)

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Johnson *et al.*(1) have shown that the young calf on a "normal" methionine intake requires a dietary source of choline and the present investigation was undertaken to determine whether this was replaceable by methionine. Earlier work has shown that rat liver slices and homogenates methylate homocysteine to form methionine with either choline or betaine as methyl donor. In studies by Borsook and Dubnoff(2), guanidoacetic acid was shown to be methylated by methionine in guinea pig liver homogenates. Bernheim and Bernheim(3) demonstrated that rat liver homogenates could oxidize acetylcholine, but found guinea pig liver to be inactive in this oxidation. Kensler and Langemann(4) reported that human and rabbit liver resembled guinea pig liver in this respect. We have now studied these enzyme systems in the calf.

Methods. Methionine-choline interrelationship. Male calves of various dairy breeds, 24 hours old, were fed a synthetic milk diet previously described by Hopper and Johnson (5) except that alpha protein was used as the protein source in the diet. Various levels of DL-methionine were added to the diet at time of feeding to give the total dietary methionine values indicated in Table I. The vitamin premix contained all the vitamins, except choline, which was a variable in the experiment. When choline was administered, it was a constituent of the vitamin premix. All animals were fed *ad lib.* twice daily from nipple pails. At the end of the trials, all animals were sacrificed and liver samples obtained for ether extract determinations. Post mortem examinations of all animals were conducted for the detection of histological lesions.

In vitro studies using calf liver homogenates. Liver samples were obtained from calves which had been fed the complete synthetic milk diet and compared with liver samples from 175-200 g, male rats fed commercial laboratory pellets. These samples were handled and the study of the methylation of homocysteine by betaine was conducted by

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† This paper is taken from a thesis submitted by the senior author to the graduate college, University of Illinois in partial fulfillment of requirements for the Degree of Doctor of Philosophy in Animal Nutrition. Present address: Department of Animal Husbandry, University of Nebraska, Lincoln.

TABLE I. Effect on Various Levels of Dietary Methionine on Growth and Liver Ether Extract of Animals on Choline-Deficient Diets, in 15 Calves.

Days on trial	Total dietary methionine, %*	Dietary choline, %	Daily gain, lb	% ether extract in liver (dry matter basis)
35	.4	0	.26	9.75
35	"	0	.19	3.65
43	"	0	.35	6.42
41	"	0	.34	4.17
38	.8	0	.66	38.96
35	"	0	.86	23.32
44	1.2	0	.91	13.65
43	"	0	.70	13.41
33	"	0	1.00	9.52
33	"	0	1.40	18.00
41	1.6	0	.34	37.68
41	"	0	.27	26.57
41	.8	.2	.90	14.04
41	"	"	.54	4.90
33	"	"	1.20	9.56

* The basal ration contained 0.3% methionine, and DL-methionine was added to give these total values.

procedures outlined by Williams *et al.*(6) and by Mistry *et al.*(7). The methylation of guanidoacetic acid by methionine was studied according to the method of Borsook and Dubnoff as used by Mistry *et al.*(7). Choline oxidase activity was determined by the method of Richert and Westerfeld(8).

Results. In vivo studies. As noted in Table I, good growth was observed in the calves receiving the choline-deficient diets containing 0.8 or 1.2% DL-methionine. However, the livers of the calves receiving only 0.8% DL-methionine contained a considerably greater amount of ether extract. This indicates a deficiency of labile methyl groups at this level of methionine in the absence of choline. Treadwell(9) has reported that the young rat has a definite need for choline when the diet contains 0.8% methionine. Neumann *et al.*(10) found the young pig required 0.1% choline when fed a "synthetic milk" ration containing 0.8% methionine. The calves receiving a total of 0.4% methionine in the diet‡ and no choline exhibited very small daily gains. This result was due to a methionine deficiency as well as a deficiency of labile methyl groups and the extremely poor growth presumably accounts for the failure to find choline-deficiency fatty livers. Diets supplying a total of 1.6% methionine supported very poor growth and liver samples from these animals during the feeding trial indicated that the 1.6% level of methionine was apparently "toxic," as the animals scoured severely throughout the trial. Liver ether extract data for the animals receiving 1.2% methionine, are similar to those from the normal animals receiving 0.8% methionine and 0.2% choline, as seen in Table I. Histological study of liver revealed fatty infiltration and some kidney damage in the methyl-deficient animals, confirming the liver fat data. The results indicate that the young calf can, by transmethylation, synthesize its required choline from methionine as has been shown for the rat, for the pig, and for man.

‡ This diet also contained approximately 0.15% cystine, supplied in the alpha protein.

In vitro studies. The results of methionine formation by calf liver homogenates are shown in Table II. Although there was some transmethylase activity in calf liver homogenates, the activity was much less than that of rat liver homogenates even though both species were on diets which were satisfactory for growth.

The results of creatine formation by calf liver homogenates are presented in Table II. Under the same experimental conditions, over 4 times as much creatine was formed by rat liver homogenates as by calf liver homoge-

TABLE II. Results of Enzyme Studies in Calf and Rat Liver Homogenates.

	No. of animals	Results
Methionine synthesis from homocysteine + betaine		Avg methionine formed/100 mg dry matter of liver tissue/hr, μg
Calf	2	59
Rat	3	88
Creatine synthesis from guanidoacetic acid + methionine		Avg creatine formed/100 mg dry matter of liver tissue/hr, μg
Calf	2	6.9
Rat	2	29.6
Choline oxidase activity		$\mu l/O_2/10$ min./flask
Calf	2	4.78
Rat	2	56.6

nates. However, both species were able to methylate guanidoacetic acid to form creatine. The low values found in this study for calf liver are of the same magnitude as those reported by Cohen(11) for beef liver. Cohen (12) found that addition of folic acid and glutamic acid did not bring the levels into line with those for the rat.

The choline oxidase activity of calf liver homogenates as compared to rat liver homogenates is shown in Table II. These results indicate that calf liver possesses a much lower choline oxidase activity than rat liver. The low activity of the enzyme would indicate a slow choline turnover similar to that found by Handler(13) for the guinea pig.

Summary. 1. Using a synthetic milk diet, calves receiving 1.2% DL-methionine exhibited no demonstrable choline requirement, as measured by growth and liver ether extract. On a diet supplying 0.8% methionine, fatty livers developed in the absence of choline. 2. A study was made of the ability of calf liver homogenates to methylate homocysteine to form methionine, when betaine served as methyl donor; to methylate guanidoacetic acid, when methionine served as the methyl donor, and to oxidize choline. It was found that while calf liver homogenates would carry out all of these reactions, the activity of the calf liver enzymes was very much less than that of rat liver homogenates.

1. Johnson, B. C., Mitchell, H. H., Pinkos, J. A., and Morril, C. C., *J. Nutrition,* 1951, v43, 37.

2. Borsook, H., and Dubnoff, J. W., *J. Biol. Chem.,* 1947, v171, 363.

3. Bernheim, F., and Bernheim, Mary L. C., *Am. J. Physiol.,* 1933, v104, 438.

4. Kensler, C. J., and Langemann, H., PROC. SOC. EXP. BIOL. AND MED., 1954, v85, 364.

5. Hopper, John H., and Johnson, B. Connor, *J. Nutrition,* 1955, v56, 303.

6. Williams, J. N., Jr., Monson, W. J., Sreenivasan, A., Dietrich, L. S., Harper, A. E., and Elvehjem, C. A., *J. Biol. Chem.,* 1953, v202, 151.

7. Mistry, S. P., Vadopalaite, I., Chang, I., Firth, J., and Johnson, B. C., *ibid.,* 1955, v212, 713.

8. Richert, D. A., and Westerfeld, W. W., *ibid.,* 1952, v199, 829.

9. Treadwell, C. R., *ibid.,* 1948, v176, 1141.

10. Neumann, A. L., Krider, J. L., James, Marian F., and Johnson, B. C., *J. Nutrition,* 1949, v38, 195.

11. Cohen, S., *J. Biol. Chem.,* 1951, v193, 851.

12. ———, *Fed. Proc.,* 1952, v11, 197.

13. Handler, P., PROC. SOC. EXP. BIOL. AND MED., 1949, v70, 70.

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Pernicious Anemia. I. Remission by Small Oral Doses of Purified Vitamin B_{12}.* (22306)

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During studies on pernicious anemia, it was found, contrary to previous experience, that partial remission occurred in a patient with pernicious anemia in relapse who received minimal doses of purified vit. B_{12} by mouth. Pernicious anemia is generally considered to be the result of a heredofamilial atrophy of gastric and duodenal mucosa, with absence of "intrinsic factor," a material necessary for absorption of vit. B_{12} from the gastrointestinal tract into the blood stream. Megaloblastic erythropoiesis of pernicious anemia in relapse can be restored to normal by overcoming the deficiency in intrinsic factor by (1) parenteral administration of vit. B_{12}, thus circumventing the abnormal gastrointestinal tract; (2) oral administration of massive doses of vit. B_{12} (1000 to 9000 μg/wk), through a sort of "mass action" effect; or (3) oral administration of vit. B_{12} together with some outside source of intrinsic factor. How-

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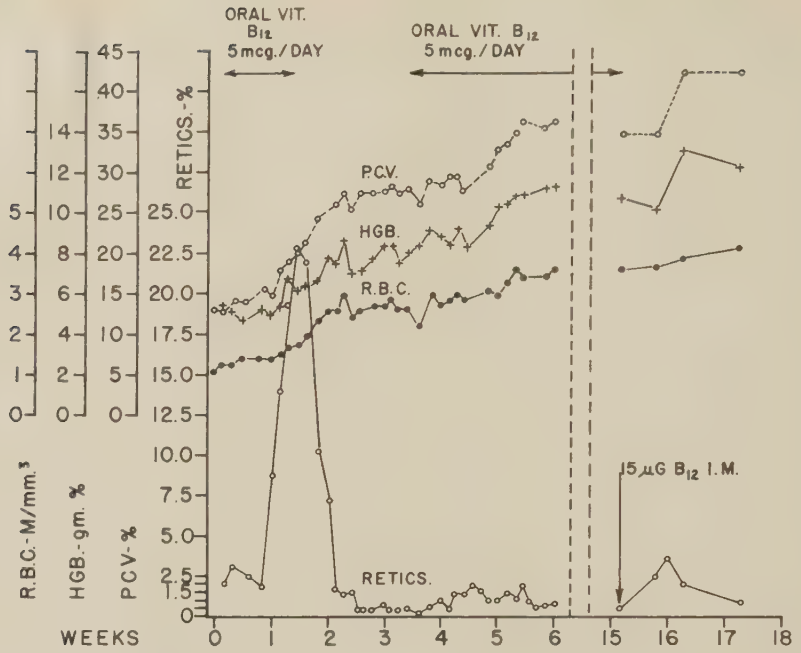

FIG. 1. Case 1a. Pernicious anemia. Submaximal response following oral administration of vit. B_{12}, 5 μg daily.

ever, remissions of pernicious anemia following oral administration of small amounts of vit. B_{12} without intrinsic factor (10 to 250 μg/day) have been reported(1-7). Suboptimal responses have been seen with oral doses of 10 μg(1,2), 15 μg(3), 30 μg(2), and 50 to 250 μg per day(1,4-6); but only rarely have maximal responses to oral vit. B_{12} in doses of 25 to 150 μg/day been noted(1,4,6). In the absence of an outside source of intrinsic factor, remission following oral vit. B_{12} occurred regularly only when massive doses of the material (3000 μg in a single dose) were given (8).

As a result of our experience with one patient (Fig. 1), in whom pronounced reticulocytosis and remission followed oral administration of 5 μg/day of purified vit. B_{12} without added intrinsic factor, similar small doses of vit. B_{12} (5.0 to 16.8 μg/day) were given by the same route to 9 additional patients with pernicious anemia in relapse, and to 1 patient with megaloblastic anemia of obscure etiology. It is our purpose to report the almost regular occurrence of varying degrees of remission under these circumstances.

Methods. Diagnosis of pernicious anemia was established by the presence of macrocytic anemia, histamine-fast achlorhydria, megaloblastic bone marrow, normal gastrointestinal x-ray studies, and absence of steatorrhea. The dose of 5.0 to 16.8 μg of vit. B_{12} was administered by mouth, one to 3 times daily, in tablets or capsules, with no particular relation to meals(6). The vit. B_{12} was obtained from 2 independent sources,†, and was assayed for and found to be free of folic and folinic acids. No intrinsic factor was given in association with oral vit. B_{12}, and the diet, which was free of liver and red meats, was unchanged before and during treatment.

Results. A definite reticulocytosis and hematologic remission of varying degree, with reversion of bone marrow to normoblastic erythropoiesis, occurred in 11 trials in 8 cases of pernicious anemia and in one case of non-pernicious megaloblastic anemia (Table I).

† We are indebted to Dr. Douglas B. Remsen, Squibb Institute for Medical Research, New Brunswick, N. J., and to Dr. Nathaniel S. Ritter, Merck & Co., Rahway, N. J., for generous supplies of these materials.

TABLE I. Remissions in Pernicious Anemia (Cases 1-8) and Non-Pernicious Megaloblastic Anemia (Case 9) on Small Oral Doses of Vit. B_{12}.

Case	B_{12}/day, μg	Initial count: Hgb, g/100 ml	Initial count: Hct, %	Max retics, %/day	Max hemogram: Hgb, g/100 ml	Max hemogram: Hct, %
1 a	5	5.2	13	22.6/ 8	11.4	37
b	10	9.0	27	6.2/ 9	12.6	42
2 a	16.8	5.6	18	9.8/ 3	10.9	37
b	15	8.0	26	9.2/ 8	10.7	36
3	16.8	9.1	27	9.2/ 9	13.3	41
4	15	8.2	26	8.8/20	14	42
5 a	15	8.5	28	9.8/ 9	14.4	43
b	15	9.0	28	8.8/13	14.9	47
6	15	7.6	22	18.0/ 8	11.7	37
7	15	5.4	18	21.0/ 9	10.2	34
8	10	9.3	30	7.4/12	14.1	46
9	10	5.4	17	21.6/15	12.9	46
	Mean*	7.7	23.9	10.7/10	12.6	40.2
	Range*	5.2–9.3	13–28	6.2–22.6/3–20	10.2–14.9	34–47

* Of cases 1-8 only.

Remission was estimated to have been maximal in 3 cases of pernicious anemia and in the one case of non-pernicious megaloblastic anemia, and submaximal in 5 cases of pernicious anemia. Typical responses to oral doses of vit. B_{12} are depicted in Fig. 1-3, and the 9 responsive cases are summarized in Table I. Maximal reticulocytosis usually occurred on 8th or 9th day after initiation of therapy, with a cumulative oral dose of from 35 to 150 μg of vit. B_{12}. Unless parenteral B_{12} was subsequently administered, remission after cessation of therapy usually tended to be short-lived (4 to 6 months), although remissions persisted longer in some patients.

During these observations, 3 patients with pernicious anemia similarly treated failed to show hematologic response (Table II). Case 4 initially showed no hemoglobin response, despite a reticulocytosis, on a dose of 5.6 μg/day, but responded maximally on a dose of 15 μg/day (Table I). Six months later, when again in relapse, this patient failed to show any signs of remission on oral vit B_{12} during

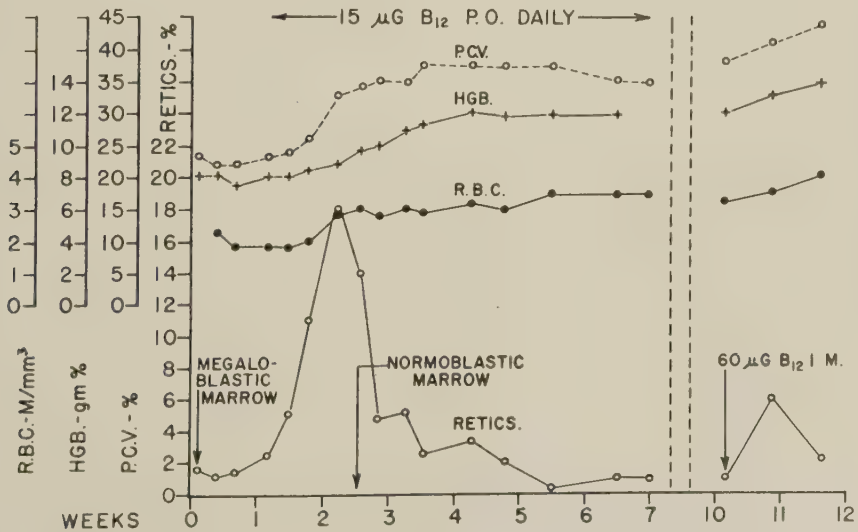

FIG. 2. Case 6. Pernicious anemia. Submaximal response following oral administration of vit. B_{12}, 15 μg daily.

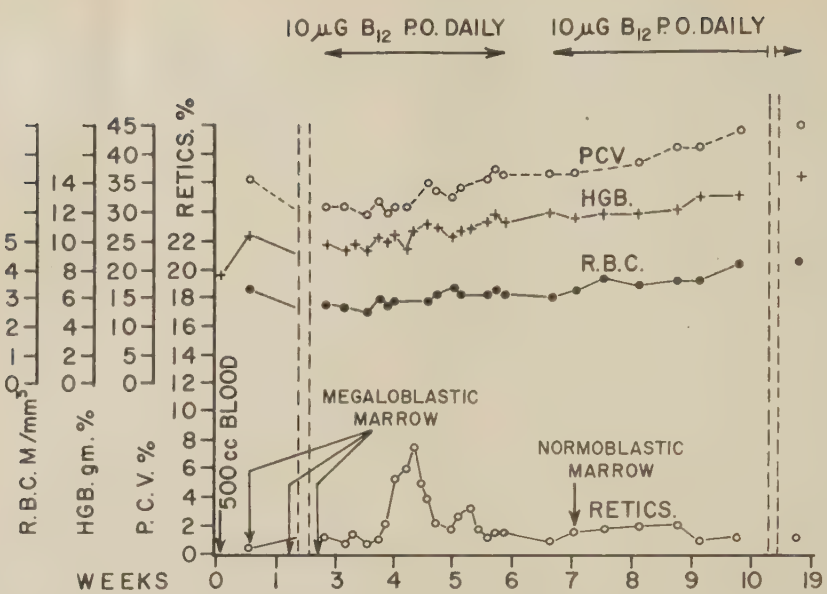

FIG. 3. Case 8. Pernicious anemia. Maximal response following oral administration of vit. B_{12}, 10 μg daily.

successive trials on 5, 10 and 15 μg/day, but at this time he had developed carcinoma of the stomach. Case 7, who showed a remission on the initial oral medication of 15 μg daily (Table I), failed to respond to a similar oral dose following another relapse 9 months later (Table II), whereas parenteral vit. B_{12} was effective. No response to 5 μg of vit. B_{12} was noted in one case of pernicious anemia in relapse (Case 10, Table II), but a larger dose and longer period of observation were not attempted in this patient.

Discussion. If intrinsic factor is indeed necessary for absorption of vit. B_{12}, it appears that some intrinsic factor must have been present in gastric and duodenal mucosa of our patients with pernicious anemia in relapse. Similar conclusions regarding the quantitative rather than qualitative change in gastric secretion in some cases of pernicious anemia were reached by others(9,10), who suggested that the red cell level in pernicious anemia is determined by actual amount of intrinsic factor secreted in the gastric juice.

Since crystalline vit. B_{12} was used in these studies, it is recognized that absorption may have been facilitated by the purified nature of the material, just as ferrous sulfate is absorbed more easily than food iron. This, however, is not a satisfactory explanation in itself, since regular remission with oral use of purified vit. B_{12} has not been reported(1-7).

TABLE II. Cases of Pernicious Anemia Which Failed to Respond to Oral Vit. B_{12}.

Case	B_{12}/day, μg	Initial count: Hgb, g/100 ml	Initial count: Het, %	Max retics, %/day	Max hemogram: Hgb, g/100 ml	Max hemogram: Het, %
4*	5.6	5.3	16.5	6.6/ 8	6.4	20
	5	8.4	27	4.8/18	8.4	27
	10	6.9	27	5.0/ 4	7.2	24
	15	7.0	23	5.4/26	7.0	23
7*	15	6.6	20.5	2.6/16	6.6	20.5
10	5	8.2	25	2.1/ 8	7.4	25
	Mean	7.1	23.2	4.4/13	7.2	23.3
	Range	5.3–8.4	16.5–27	2.1–6.6/4–26	6.4–8.4	20–27

* Same numbers as in Table I.

Although the fasting state may facilitate absorption of vit. B_{12}(6) from the gastrointestinal tract, absorption occurred independent of the fasting state in our patients.

The action of oral preparations containing both intrinsic factor and vit. B_{12} cannot *a priori* be attributed to their intrinsic factor content, since as has been shown above, remission may follow the use of the vit. B_{12} alone.

Studies on vit. B_{12} metabolism in this group of patients are still in progress. Preliminary results in some of these patients suggest that remission occurs without a rise of the serum vit. B_{12} level above relapse levels (less than 20 $\mu\mu$g/ml).

Summary. 1. Hematologic and clinical remissions were induced 11 times in 8 patients with pernicious anemia in relapse, and in 1 patient with non-pernicious megaloblastic anemia, by small oral doses of purified vit. B_{12} without added intrinsic factor. The effective dosage range was 5 to 16.8 μg per day. 2. Unequivocal failure occurred in 1 case of relapsed pernicious anemia. 3. These results further confirm the suggestion that in pernicious anemia there is a quantitative rather than a qualitative change in gastric secretion, and that variations in the amounts of intrinsic factor remaining in pernicious anemia patients may be responsible for varying responses to oral vit. B_{12}. 4. A trial period of vit. B_{12} alone should precede any study with a vit. B_{12} plus intrinsic factor preparation.

1. Hall, B. E., *Brit. Med. J.*, 1950, v2, 585.

2. Glass, G. B. J., Boyd, L. J., Rubinstein, M. A., and Svigals, L. S., *Science,* 1952, v115, 101.

3. Glass, G. B. J., and Boyd, L. J., *Blood,* 1953, v8, 867.

4. Meyer, L. M., Sawitsky, A., Cohen, B. S., Krim, M., and Fadem, R., *Am. J. Med. Sc.,* 1950, v220, 604.

5. Ungley, C. C., *Brit. Med. J.*, 1950, v2, 905.

6. Chalmers, J. N. M., and Hall, Z. M., *ibid.*, 1954, v2, 1179.

7. Spies, T. D., Stone, R. E., Lopez, G. G., Milanes, F., Toca, R. L., and Aramburu, T., *Lancet,* 1954, v257, 454.

8. Conley, C. L., Krevens, J. R., Chow, B. F., Barrows, C., and Lang, C. A., *J. Lab. Clin. Med*, 1952, v38, 84.

9. Goldhamer, S. M., *Am. J. Med. Sc.,* 1936, v191, 405.

10. Campbell, D. C., Hall, B. E., and Morgan, E. H., *J. Lab. Clin. Med.,* 1949, v34, 1590.

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Δ^1-Hydrocortisone: Plasma 17-Hydroxycorticosteroid Concentrations Following Oral and I. V. Administration.* (22307)

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During the past year the Δ^1-steroids have been employed clinically to a considerable extent. These compounds are synthetic derivatives of hydrocortisone and cortisone, differing from the parent steroids by the insertion of a double bond in the 1-2 position of the steroid nucleus. Δ^1-hydrocortisone ($\Delta^{1,4}$-pregnadiene-11β, 17α, 21-triol-3,20-dione) has been produced under various commercial names: metacortandralone, prednisolone, delta-cortef, hydeltra, meticortelone, and sterane.

Clinically, the Δ^1-steroids appear to be 3 to 5 times as effective as the parent compounds(1-3). A similar ratio has been found for glucocorticoid activity (liver glycogen deposition, eosinophil response)(3,4). However, results of *in vitro* studies of anti-inflammatory potency have not been reported. It is possible that the apparent advantages of the Δ-steroids over the parent compounds are attributable to differences in absorption or

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metabolism which permit more efficient utilization of the former steroids. This possibility has been investigated in the present study by measuring in human subjects the influence of oral or I. V. Δ^1-hydrocortisone administration on plasma 17-hydroxycorticosteroid (17-OHCS) concentrations.

Materials and methods. Normal young adults were the subjects used in all the following studies. In the study of oral administration each of 9 subjects was given a dose of 45 mg Δ^1-hydrocortisone§ orally. Plasma samples were obtained before and at 1, 2, 4, 6, and 8 hours after administration of the test dose and 17-OHCS concentrations determined by the method of Nelson and Samuels(5,6), using hydrocortisone as the reference standard. In another group of 6 subjects each was given a dose of 40 mg free hydrocortisone‖ orally and plasma samples were obtained for 17-OHCS determinations at 0, 2, 4 and 6 hours. In a third group of 11 subjects each was given 100 mg free hydrocortisone orally, and plasma samples were obtained at 0, 1, 2, 4 and 8 hours for 17-OHCS determinations. In the study of I. V. administration each of 8 subjects was given a dose of 0.5 mg/kg Δ^1-hydrocortisone intravenously as a 125 ml infusion over 30 minutes. Plasma samples for 17-OHCS concentration were obtained 1, 2, 3, and 4 hours after administration. From these data a regression line of best fit was determined by the method of least squares and the 17-OHCS half-life value calculated from the slope of the line.

Results. To determine whether the Δ^1-steroid was measured quantitatively by the Nelson and Samuels technic a comparison of the color development with the phenylhydrazine-sulfuric acid reagent was made using standard hydrocortisone and Δ^1-hydrocortisone. The resulting absorption curves for the two steroids were similar over a wave length range of 340-480 mμ, differing in that the

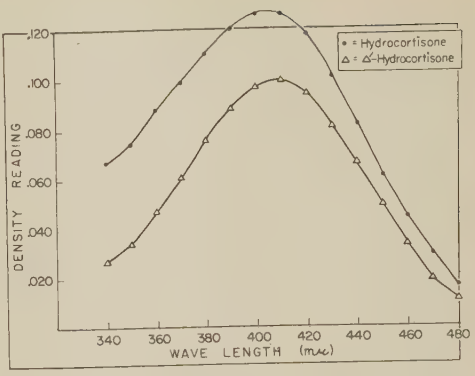

FIG. 1. Δ^1-hydrocortisone vs hydrocortisone: Absorption curves using phenylhydrazine-sulfuric acid reagent.

curve for hydrocortisone had a greater density throughout (Fig. 1). After a correction factor for background influence was applied the density values at 410 mμ were essentially superimposed. The density values at 410 mμ for different amounts of steroid (Fig. 2) were shown to be the same for Δ^1-hydrocortisone as for hydrocortisone. Also, recovery following chromatography was almost the same, averaging 70% for Δ^1-hydrocortisone and 76% for hydrocortisone.

In Fig. 3 is shown a comparison of mean plasma 17-OHCS values at various time intervals following the oral administration of Δ^1-hydrocortisone and of free hydrocortisone. Following 45 mg Δ^1-hydrocortisone the peak 17-OHCS concentrations were greater than those following 40 mg free hydrocortisone, but less than those following 100 mg free hydrocortisone. In those subjects given 40

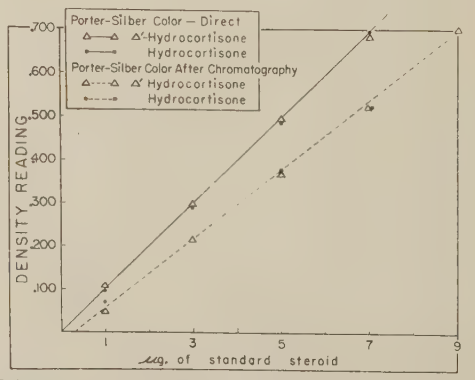

FIG. 2. Δ^1-hydrocortisone vs hydrocortisone: phenylhydrazine-sulfuric acid color development (410 mμ) directly and after column chromatography.

§ C. T. Delta-Cortef® supplied through courtesy of Dr. C. J. O'Donovan, Upjohn Co., Kalamazoo, Mich.

‖ C. T. Cortef® supplied through courtesy of Dr. C. J. O'Donovan, Upjohn Co., Kalamazoo, Mich.

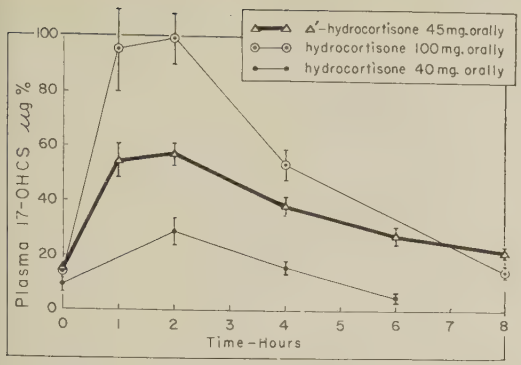

FIG. 3. Δ^1-hydrocortisone vs hydrocortisone: Plasma 17-OHCS concentrations following oral administration.

mg free hydrocortisone orally the plasma 17-OHCS concentrations were at or below control levels by 6 hours. A similar decline is seen in the curve representing the plasma 17-OHCS concentrations following the administration of 100 mg hydrocortisone orally, a return to normal values occurring by 8 hours. In contrast, the Δ^1-hydrocortisone curve falls more slowly; at 8 hours the mean 17-OHCS concentration is still well above the control value—and is significantly higher than the corresponding value following 100 mg hydrocortisone ($p < .01$).

The influence of intravenous administration of Δ^1-hydrocortisone on plasma 17-OHCS concentrations is indicated by the half-life data in Table I. The mean half-life obtained in 8 subjects was 192 minutes. In contrast to this, data reported from this laboratory(7) and elsewhere(8,9) for the mean half-life of hydrocortisone indicate that this is significantly less, ranging from 90 to 115 minutes. Thus, intravenous administration of Δ^1-hydrocortisone produces a more prolonged elevation of plasma 17-OHCS than does the intravenous administration of hydrocortisone.

Discussion. It has been shown that orally administered Δ^1-hydrocortisone, in doses roughly equivalent to those of free hydrocortisone, caused plasma 17-OHCS elevations both of greater magnitude and longer duration than did the latter steroid. When the dose of hydrocortisone was increased 2½ times the magnitude of 17-OHCS elevations became greater than that following Δ^1-hydrocortisone. However, even following the administration of this larger dose, these elevations disappeared more rapidly than those following the Δ^1-steroid. In addition, the 17-OHCS half-life in subjects receiving Δ^1-hydrocortisone intravenously was more prolonged than in those receiving hydrocortisone. These observations indicate that no appreciable differences in absorption exist but that the metabolism of Δ^1-hydrocortisone proceeds at a rate slower than that of hydrocortisone.

Results of *in vitro* experiments(10,11) are in accord with this interpretation. The biologically active adrenal corticosteroids contain the Δ^4-3-ketone structure in ring A, and in their metabolism this ring is reduced at carbons 4, 5, and then 3. The Δ^1-steroid must first undergo saturation of the 1-2 double bond to form the Δ^4 compound. Only following this is the Δ^4-3-ketone reduced to form the tetrahydro steroid and subsequently conjugated.

These data from both *in vivo* and *in vitro* experiments lend support to the possibility that differences in the rate of metabolism may account for the equivalent clinical effectiveness of smaller doses of the delta forms than of hydrocortisone or cortisone. To date, Δ^1-hydrocortisone represents the only steroid

TABLE I.

Plasma 17-OHCS Half-Life Values following Intravenous Δ^1-Hydrocortisone.

Normal patients	Half-life (min.)
D.C.	217
F.B.	189
T.H.	141
E.H.	205
J.K.	185
J.N.	232
L.O.	171
A.D.	197
Mean	192 ± 10

Plasma 17-OHCS Half-Life following Intravenous Hydrocortisone.

Author reference	No. normals	Mean half-life (min.)
Done(7)	9	90 ± 7.4
Brown(8)	11	111 ± 5.3*
Peterson(9)	18	115

* Calculated from author's individual 17-OHCS data.

which has been reported to have a half-life longer than that of hydrocortisone. Others studied include tetrahydro-hydrocortisone, 20-hydroxy-hydrocortisone, cortisone, and corticosterone(8,9); each of these has a half-life shorter than that of hydrocortisone. Of the steroids mentioned only cortisone, hydrocortisone, and Δ^1-hydrocortisone have a Δ^4-17, 21-dihydroxy-3,20-keto configuration and are potent glucocorticoids. Among these 3, the glucocorticoid potency and the half-life are related in such a way as to suggest that glucocorticoid potency is inversely proportional to the rate of metabolism.

Summary. The influence of oral or intravenous administration of Δ^1-hydrocortisone on plasma 17-OHCS concentrations was studied. With comparable oral doses Δ^1-hydrocortisone produced 17-OHCS elevations of greater magnitude and longer duration than did hydrocortisone. Also, the half-life of the Δ^1-steroid was longer than that of hydrocortisone. It was concluded that a slower metabolism of the Δ^1 form occurs and postulated that this might contribute to the greater clinical effectiveness of this steroid.

1. Bunim, J. J., Pechet, M. M., and Bollet, A. J., *J. Am. Med. Assn.,* 1955, v157, 311.

2. Spies, T. D., Stone, R. E., Lopez, G. G., Tellechea, C. M. D., Toca, R. L., Reboredo, A., and Suarez, R. M., *ibid.,* 1955, v159, 645.

3. Thorn, G. W., Renold, A. E., Morse, W. I., Goldfien, A., and Reddy, W. J., *Ann. Int. Med.,* 1955, v43, 979.

4. Perlman, P. L., and Tolksdorf, S., *Fed. Proc.,* 1955, v14, 377.

5. Nelson, D. H., and Samuels, L. T., *J. Clin. Endocrinol. and Metab.,* 1952, v12, 519.

6. Eik-Nes, K., Nelson, D. H., and Samuels, L. T., *ibid.,* 1953, v13, 1280.

7. Done, A. K., Ely, R. S., Olsen, L. J., and Kelley, V. C., *Metabolism,* 1955, v4, 416.

8. Brown, H., Willardson, D. M., Samuels, L. T., and Tyler, F. H., *J. Clin. Invest.,* 1954, v33, 1524.

9. Peterson, R. E., and Wyngaarden, J. B., *Ann. N. Y. Acad. Sc.,* 1955, v61, 297.

10. Tompkins, G., and Isselbacher, K. J., *J. Am. Chem. Soc.,* 1954, v76, 3100.

11. Isselbacher, K. J., and Tompkins, G., (presented at Laurentian Hormone Conference, 1955).

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A Non-hypophyseal Sex Difference in Estrous Behaviour of Mice Bearing Pituitary Grafts.* (22308)

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Differences in size of the pituitary gland related to sex have been described in some species, the female gland being larger and heavier than that of the male. Similarly, the gonadotrophic potency of injected hypophyseal extracts from males is greater than those prepared from female pituitaries(1,2). Castrated male guinea pigs with grafted ovaries showed a greater stimulation of the mammary glands and other sexual structures, than intact females(6). The same was true in castrated male mice bearing ovarian and vaginal grafts. There was a continuous non-cyclic stimulation of the vaginal tissue(5). However, Harris and Jacobson(3,4) have demonstrated that hypophysectomized female rats bearing male hypophyseal grafts from newborn donors, had normal estrous cycles, became pregnant and delivered their young at term. This would indicate that the characteristic cyclic gonadal function of the female, as compared to the non-cyclic of the male, would not be related to the sex of the pituitary, but rather to a sex difference in some other structure. Since functional pituitary grafts can be placed in hypophysectomized mice by means of a rather simple one-stage operation, we have

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used this animal preparation to investigate further the problem of estrous behaviour in hypophysectomized females and males bearing either male or female hypophyseal grafts, respectively.

Material and methods. Young mice of both sexes of the Z (C3H) stock and AZF_1 hybrids (A ♀ x Z ♂) were used. Hypophysectomy was performed according to the technic described by Thomas(7). The grafting of hypophyseal tissue was done as follows: The donor was killed by ether, the scalp and brain were lifted and the gland was removed and placed in a container with saline. Upon completion of hypophysectomy, the gland to be grafted was transferred to the emptied sella turcica of the host either with a fine pair of forceps or by holding the gland at the distal end of a very thin glass pipette by applying a slight negative pressure. The entire procedure was completed within 4 to 5 minutes and postoperative mortality did not exceed 10%. By this procedure, the overall incidence of successful hypophyseal grafts was 85% of a total of 325 operated animals for other studies. Mice bearing hypophyseal successful grafts have shown normal sexual activity throughout their lives (12 to 16 months). Two types of experiments were done. In one, the body weight changes were determined and the sexual cycles studied in 4 groups of female mice of the Z(C3H) strain, as follows: a) hypophysectomized; b) sham operated controls; c) hypophysectomized bearing a hypophyseal graft taken from a female donor of the same strain and age; d) hypophysectomized bearing a hypophyseal graft from a male donor of the same strain and age. In all groups the body weight was determined before and at weekly intervals after the operation. Similarly vaginal smears by the douche technic were taken daily during 1 week, at monthly intervals to determine the presence or absence of estrous cycles in the different groups. In another experiment, 2 groups of male AZF_1 hybrid mice were used. One of them was submitted to hypophysectomy followed by the graft of one hypophysis taken from a female donor. Another group carried their own (male) hypophysis. Three weeks after the operation, both groups of mice were castrated and 2 ovaries and a ring of vaginal tissue taken from female mice of the same strain and age were grafted subcutaneously, using the technic described by Huseby and Bittner(5). Finally, after a period of one month following the vaginal transplant, mice were killed, the grafts removed and studied histologically. The presence of hyperplasia and hypertrophy of the epithelial wall of the vaginal graft, and a complete cornification of the content of the lumen, without signs of cyclic activity, was interpreted as indicative of a continuous estrogenization(5).

Results. The results obtained in the first experiment, in which only animals showing successful takes of the grafts were included, are listed in Table I and can be summarized as follows: a) Sham hypophysectomized controls showed a progressive increase in body weight after the operation and, as was expected, normal estrous cycles. b) Hypophysectomized alone showed a progressive decrease in body weight and absence of estrous cycles. c) In both groups of hypophysecto-

TABLE I. Effect of Sex of Pituitary Gland Grafted into Hypophysectomized Female Mice on Body Weight and on Estrous Cycles.

Group	No. of mice	Body wt in g (mean)			Presence of estrous cycles*
		Before operation	1 mo after	2 mo after	
Sham operated controls	15	16.50 ± .38	19.25 ± .39	22.90 ± .42	Yes 15/15
Hypophysect. bearing ♀ pituitaries	15	15.91 ± .41	17.62 ± .44	18.94 ± .39	" 15/15
Hypophysect. bearing ♂ pituitaries	22	16.20 ± .36	17.90 ± .34	19.05 ± .28	" 22/22
Hypophysectomized	12	16.80 ± .45	15.10 ± .50	15.00 ± .49	No 12/12

* Normal estrous cycles in animals bearing pituitary grafts appeared at 10 to 15 days following operation.

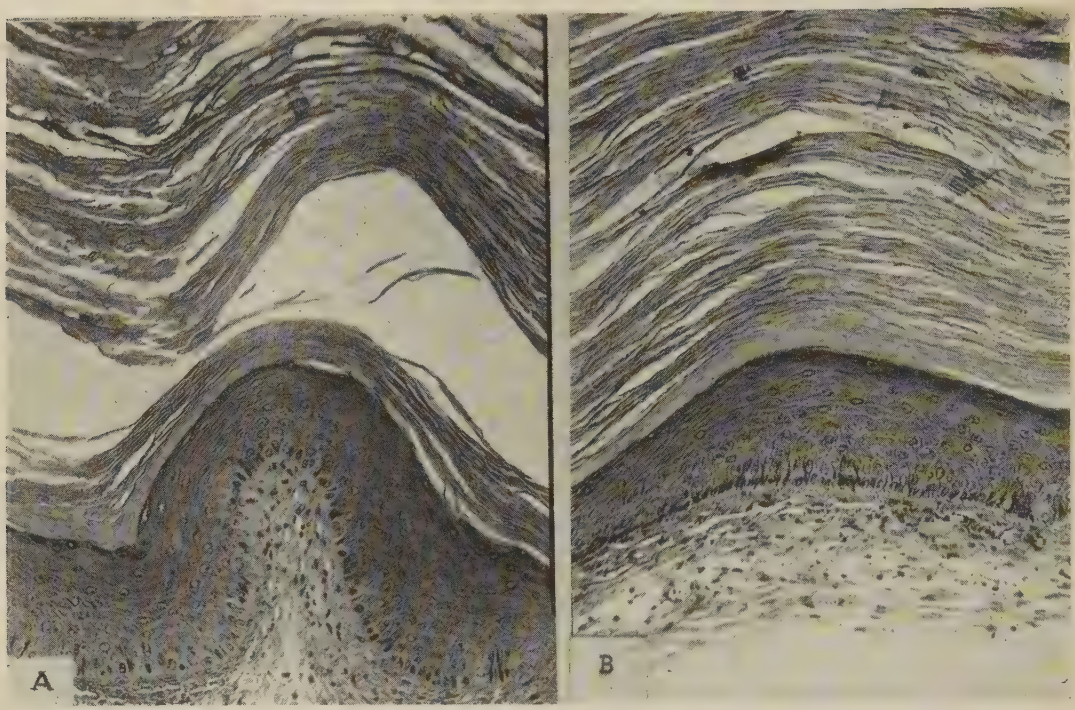

FIG. 1. Histological sections taken from vaginal grafts in castrated male mice with grafted ovaries, bearing either a female (A) or a male (B) hypophysis.

mized females bearing either a female or male hypophyseal gland, progressive increase in body weight and normal estrous cycles were observed.

Table II shows the results of the second experiment. Both groups of castrated males with grafted ovaries showed the typical non-cycling vaginal reaction characteristic of a continuous estrogenization, regardless of the sex of the pituitary gland grafted (Fig. 1).

Discussion. From the results described, it seems clear that hypophysectomized female mice bearing either female or male pituitaries had normal estrous cycles. These results agree with those obtained by Harris and Jacobson(3,4) in the rat. Furthermore, castrated male mice with grafted ovaries showed a continuous noncyclic vaginal stimulation regardless of the sex (female or male) of the hypophyseal tissue grafted.

TABLE II. Effect of Sex of Pituitary Gland on Development of Vaginal Grafts in Castrated Males with Grafted Ovaries.

Group	Pituitary	No. of mice	Histology of vaginal grafts
Castrated with grafted ovaries	♂ (own)	10	Cornified non-cyclic 10/10
Idem	♀	8	*Idem*
"	None	2	Atrophic or reabsorbed

These results would imply that the normal cyclic function of the female pituitary as opposed to the non-cyclic of the male, as it was formerly described, would not reside in a sex difference of the hypophyseal tissue in itself, for in our experiments, the property of cycling or non-cycling seemed to be determined by the sex of the host, rather than that of the pituitary gland they carried.

In the light of these data it is rather difficult to decide as to where and how the sex difference in gonadal function of the hypophysis originates. Not being involved, as it appears, the sex of the hypophyseal gland, and since anatomically this organ is related to the hypothalamus in the brain through vascular and nervous connections, it would be perhaps justified to assume that the cyclic or non-cyclic character of the female or male gonadotrophic function might depend upon a

sex difference of that nervous structure.

Summary. Estrous behaviour in hypophysectomized female and male mice bearing hypophyseal grafts has been studied. The results were: (1) Hypophysectomized female mice bearing either female or male hypophyseal grafts, showed complete and normal estrous cycles. (2) Castrated males bearing ovarian and vaginal grafts showed a continuous noncyclic type of vaginal stimulation regardless of whether they had been grafted with male or female pituitaries. (3) The characteristic cyclic function of the female hypophysis may be related to the cyclic function of some other controlling structure rather than being dependent upon the pituitary gland itself.

1. Domm, L. V., PROC. SOC. EXP. BIOL. AND MED., 1931, v29, 308.

2. Evans, H. M., Simpson, M. E., *Am. J. Physiol.*, 1929, v89, 375.

3. Harris, G. W., Jacobson, D., *J. Physiol.*, 1951, v113, 35P.

4. ———, *Ciba Foundation Colloquia in Endocrinology*, 1951, vIV, 115.

5. Huseby, R. A., Bittner, J. J., PROC. SOC. EXP. BIOL. AND MED., 1948, v69, 321.

6. Smelser, G. K., *Physiol. Zool.*, 1933, v6, 396.

7. Thomas, F., *Endocrinology*, 1938, v23, 99.

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Threshold of the Enterogastric Reflex.* (22309)

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Introduction of acid into the duodenum causes a prompt and complete inhibition of gastric motility. This phenomenon has been named by Thomas, *et al.*, the "enterogastric reflex"(1). In a previous study, we reported this inhibition in the human subject and found that it occurred in patients with vagotomy, extensive sympathectomy, partial gastric resection, and spinal cord transection(2). It is the purpose of the present study to explore the threshold of this inhibitory mechanism and to determine its presence in patients with gastric achlorhydria.

Method. As previously described(2), a duodenal tube was passed under fluoroscopic control. A second tube with an attached balloon was passed into the antral portion of the stomach, and gastric motility was recorded. Studies were performed on 11 normal human subjects, varying both the volume and concentration of hydrochloric acid solutions instilled via the duodenal tube. In most patients, 2 or more determinations were made during the same study period—a total of 25 studies. In addition, the same study was performed on 3 patients with persistent gastric achlorhydria as determined by histamine stimulation.

Results. 1. *0.075 N HCl*: In 4 patients, 100 cc of 0.075 N HCl introduced into the duodenum caused a prompt inhibition of gastric motility varying from 26 to 39 minutes. Twenty-five cubic centimeters of the same solution in one patient caused only a 10 minute period of inhibition. 2. *0.05 N HCl*: One hundred cubic centimeters of 0.05 N HCl infused into the duodenum of 5 subjects resulted in cessation of gastric motor activity varying from 9 to 26 minutes. In 2 patients, 25 cc of the same solution instilled into the duodenum caused a 5 and a 6 minute inhibition, respectively. 3. *0.025 N HCl*: One hundred cubic centimeters of 0.025 N HCl was introduced into the duodenum of 3 subjects and was immediately followed by inhibition of gastric motility lasting for 9 to 16 minutes. In 1 patient, 25 cc of this solution produced a 10-minute period of inhibi-

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† John and Mary R. Markle Scholar in Medical Science.

tion. 4. *0.01 N HCl*: One hundred cubic centimeters of 0.01 N HCl was instilled into the duodenum of 3 patients and resulted in a gastric motor inhibition lasting 5 to 8 minutes. In 1 patient, 50 cc of this solution inhibited gastric activity for 5 minutes; in 2 patients, 25 cc of the same solution inhibited gastric contractions for only 3 minutes.

In the 3 patients with persistent histamine achlorhydria, 0.1 N HCl instilled into the duodenum resulted in prompt and profound inhibition of gastric motility. It was indistinguishable in latent period, degree, and duration from that occurring in subjects with normal gastric secretion.

Conclusions. 1) Duration of inhibition of gastric motility varies directly with volume and concentration of HCl solutions introduced into the duodenum. 0.075 N HCl produces definitely shorter inhibition than was previously found with equal volumes of 0.1 N HCl. When concentration of HCl is reduced to 0.01 N and volume reduced to 25 cc, the inhibitory period is very brief, lasting only 3 minutes. 2. In 3 patients with complete histamine achlorhydria, the inhibitory effect of intraduodenal acid on gastric motility was the same as that in individuals with normal gastric secretion.

1. Thomas, J. E., Crider, J. O., and Morgan, C. J., *Am. J. Physiol.*, 1934, v108, 683.

2. Schapiro, H., and Woodward, E. R., *J. Appl. Physiol.*, 1955, v8, 121.

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Organ-Specific Effects of Cortisone on Protein Synthesis in Protein Depleted Rats.* (22310)

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The mechanism of action of cortisone and other adrenocortical steroids on protein metabolism has been the subject of many investigations. The theories and observations of some investigators(1-3) seem to favor a primary anti-anabolic effect while other workers(4-6) favor a primary catabolic mechanism. Still other reports(7) support a more complex interpretation.

Previous work(8) indicated that rats maintained on a completely protein-free diet offered a unique situation in which to study the effects of cortisone. The present communication is a preliminary report of results obtained from a study of the incorporation *in vitro* of glycine-2-C^{14} into protein fractions of liver slices and intact isolated diaphragms from protein depleted rats treated with cortisone. The liver and diaphragm were studied in the hope that they might show differences in their ability to incorporate labeled amino acids and that these changes would reflect the influence of cortisone on protein synthesis or breakdown.

Material and methods. Young male rats were maintained on 3 experimental regimens (8). These regimens included: 1) normal synthetic diet; 2) protein-free synthetic diet; 3) protein-free synthetic diet plus daily subcutaneous injections of 2.5 mg of cortisone/100 g body weight. All groups were maintained on the experimental regimens for 12 to 17 days. Following the period of treatment the rats were lightly anesthetized with ether and sacrificed by exsanguination from the heart. The livers and diaphragms were removed, weighed, and placed immediately in chilled Krebs-Hensleit media equilibrated with 95% oxygen and 5% CO_2. The incu-

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TABLE I. *In Vitro* Incorporation of Glycine-2-C^{14} into Protein Fractions of Rat Liver Slices and Intact Isolated Diaphragms. (Values are expressed as the mean of the individual values ± the standard error of the mean.)

Treatment	Incorporation of glycine-2-C^{14} into LIVER protein fractions		Incorporation of glycine-2-C^{14} into DIAPHRAGM protein fractions	
	Without added non-labeled amino acids (cpm/mg protein)	With added non-labeled amino acids* (cpm/mg protein)	Without added non-labeled amino acids (cpm/mg protein)	With added non-labeled amino acids* (cpm/mg protein)
Normal controls	260 ± 37.2 (4)†	No observations	38 ± 5.7 (3)	36 — (1)
Protein-free diet controls	128 ± 9.0 (7)	201 ± 11.7 (5)	41 ± 5.6 (7)	60 ± 10.7 (5)
Protein-free diet + cortisone	164 ± 7.0 (7)	149 ± 11.9 (5)	61 ± 3.8 (7)	82 ± 6.6 (5)

* Final concentration of L-isomer, 10 μg/ml.
† The No. of animals in which duplicate observations were made are shown in parentheses.

bation and isolation of the protein fraction was essentially as described by Krahl(9) except for minor technical changes. Approximately 400 mg of liver slices or one hemidiaphragm was placed in screw-cap incubation bottles. These bottles contained Krebs-Hensleit media (liver, 2 ml; diaphragm, 1 ml) and glycine-2-C^{14}, 11.6 μg/ml (specific activity, 1.25 mc/mM). A mixture of non-labeled amino acids was also added to some of the flasks in addition to glycine-2-C^{14}. This mixture contained l-cysteine, dl-lysine, l-histidine, dl-leucine, dl-threonine, dl-phenylalanine, dl-valine, l-arginine, dl-methionine, dl-isoleucine, and dl-tryptophane and was adjusted to pH 7.0 with $NaHCO_3$. The tissues were incubated with shaking for 2 hours at 37°C. Radioactivity in protein fractions was determined in an internal gas flow counter. All samples were counted at maximum self-absorption. Protein content was determined by the method of Lowry *et al.*(10).

Results. In confirmation of our earlier findings(8) the animals on the normal synthetic diet gained weight, those on the protein-free diet lost about 20% of their initial body weight, and those on the protein-free diet plus cortisone lost 40% of their starting weight ($P<0.001$). Liver weight/body weight ratios were significantly increased ($P<0.001$) in the animals receiving the protein-free diet plus cortisone. The protein-free diet caused a 30% decrease in the mean diaphragm weight when compared to normal values ($P<0.001$). The increase in liver weight in the cortisone treated animals was due in part to an increased protein content. Whereas cortisone treatment caused no change in the diaphragm weight/body weight ratio the liver weight/body weight ratio increased. Thus while the diaphragm suffered with other tissues from the wasting effects of cortisone, the liver was spared.

Liver slices from animals on a protein-free diet showed a decreased utilization of glycine-2-C^{14} compared with that of liver from normal controls ($P = 0.01$) (Table I). Krahl (9) observed a similar effect in fasted rats. It was possible to increase utilization of glycine-2-C^{14} by the addition of a mixture of essential non-labeled amino acids ($P<0.001$). Liver slices from animals on a protein-free diet plus cortisone showed an increased utilization of glycine-2-C^{14} compared with that of liver from protein-free dieted animals ($P = 0.01$); in this case the addition of other amino acids had no stimulatory effect.

In the diaphragms, the diet had no effect on the utilization of glycine-2-C^{14} (Table I). Treatment with cortisone caused an increased utilization of glycine-2-C^{14} by the diaphragms ($P = 0.01$). Addition of non-labeled amino acids to the diaphragms from the cortisone treated animals caused a still further increased utilization of glycine-2-C^{14} ($P<0.02$).

Summary and conclusion. In these experiments cortisone had no antisynthetic effect; its effect on protein synthetic reactions was to increase synthesis in the diaphragm. Since liver is spared and diaphragm is not spared against the wasting effects of cortisone, the chief effect of cortisone according to our ob-

servations must be its influence on catabolic reactions. Thus the organ-specific effects of cortisone can be explained on the basis of increased catabolism in diaphragm but not in liver.

1. Albright, F., *Harvey Lectures,* 1942-43 v38, 123.
2. Clark, I., *J. Biol. Chem.,* 1953, v200, 69.
3. Sinex, F. M., *Fed. Proc.,* 1951, v10, 247.
4. Hoberman, H. D., *Yale J. Biol. and Med.,* 1950, v22, 341.
5. Engel, F. L., Schiller, S., and Pentz, I., *Endocrinology,* 1949, v44, 458.
6. Bondy, P. K., Engel, F. L., and Farrar, B., *ibid.,* 1949, v44, 476.
7. Roberts, S., *J. Biol. Chem.,* 1953, v200, 77.
8. Clark, J. H., Jr., and Pesch, L. A., *J. Pharm. and Exp. Ther.,* in press.
9. Krahl, M. E., *J. Biol. Chem.,* 1953, v200, 99.
10. Lowry, O. H., Roseborough, N. J., Farr, A. L., and Randall, R. J., *ibid.,* 1951, v193, 265.

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Effect of Serotonin on Renal Excretion of Sodium; Possible Relation to Rauwolfia Action.* (22311)

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The administration of reserpine has been found to be associated with a decrease in the concentration of serotonin in brain, platelets and intestines(1). It has also been demonstrated that rauwolfia alkaloids can produce occasional salt and water retention even to the point of congestive failure(2). The present study was undertaken to investigate the possibility that this effect of rauwolfia might be mediated through serotonin.

Materials and methods. Forty convalescent patients free of cardiovascular, renal, metabolic or endocrine disease were selected for the study. All but 3 were bed patients, and all were afebrile and on a regular diet. All were given an infusion of 250 ml of 5% glucose and water. One or 2 mg of serotonin base‡ were added to the infusion of 18 subjects, the remaining 22 serving as controls. Those receiving serotonin were unaware that any drug had been added to their infusion, and did not experience any blood pressure change or discernible local or systemic reaction. Comparable conditions and identical procedures were employed in both groups. Each subject emptied his bladder as completely as possible in the sitting or standing position. One hour after breakfast the patients voided and then drank 200 ml of water. Exactly one hour later, they again voided and were immediately given the infusion which was administered at a constant rate to require one hour for its completion. Urine specimens were obtained at the close of the infusion and at exactly hourly intervals for 2 additional hours. All hourly urine specimens, beginning with those obtained at the start of the infusion, were measured and analyzed for sodium, potassium (by flame photometer), and chloride. For the purposes of this study, the excretion rates in the hour's specimen obtained at the end of the infusion were compared with excretion rates per hour in the combined 2 hourly specimens subsequently collected.

Results. The mean excretion of sodium in mEq/hr in the control group was 7.7 at the start of the infusion, rose to 9.4 at its close, with a further increase to 9.6 in the 2-hour period after the infusion. In the serotonin group the initial value was 5.9, rose to 7.2 at

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‡ Serotonin creatinine sulfate was supplied by Dr. E. W. Young of Upjohn Co., Kalamazoo, Mich.

TABLE I. Changes in Urine Volume and Electrolyte Excretion Rates following Infusion with and without Addition of Serotonin.

	Control group					Serotonin group				
	Vol, ml/hr	Na	K	Cl		Vol, ml/hr	Na	K	Cl	
		mEq/hr					mEq/hr			
1.	57 - 68†	4.4 - 5.7	3.4 - 3.4	5.6 - 5.7	1.*	189 - 75	13.6 - 3.2	7.8 - 3.4	19.6 - 6.3	
2.	168 - 145	2.6 - 4.9	4.9 - 6.8	4.9 - 6.6	2.*	109 - 144	12.7 - 11.1	5.3 - 4.4	14.3 - 13.4	
3.	110 - 30	3.3 - 1.4	1.3 - 1.3	3.4 - 1.7	3.*	186 - 96	4.3 - 1.8	2.8 - 1.5	5.9 - 2.7	
4.	100 - 118	1.9 - 8.9	.7 - 1.8	1.8 - 7.3	4.*	62 - 155	9.1 - 6.8	1.8 - 3.6	8.8 - 8.5	
5.	78 - 76	3.0 - 3.3	3.4 - 2.9	4.7 - 3.8	5.*	59 - 102	3.5 - 3.4	2.7 - 3.7	5.5 - 5.9	
6.	40 - 28	3.2 - 1.9	2.1 - 1.6	3.8 - 2.3	6.	47 - 31	5.2 - 2.6	3.7 - 3.3	9.6 - 5.8	
7.	23 - 34	1.5 - 4.2	2.0 - 3.3	3.8 - 7.0	7.	36 - 45	5.8 - 5.4	2.2 - 3.5	6.1 - 6.5	
8.	59 - 40	11.3 - 6.8	6.0 - 2.6	11.0 - 6.1	8.	77 - 114	5.8 - 4.6	2.0 - 1.9	5.1 - 3.7	
9.	86 - 136	9.7 - 9.7	3.0 - 3.5	9.7 - 10.5	9.	57 - 39	2.4 - 3.6	1.3 - 1.5	3.0 - 4.3	
10.	129 - 134	4.4 - 5.4	2.9 - 2.7	5.4 - 5.4	10.	83 - 73	6.3 - 10.2	5.3 - 4.2	9.5 - 9.2	
11.	94 - 205	9.6 - 9.4	4.0 - 3.8	10.8 - 9.5	11.	208 - 185	6.2 - 4.4	5.6 - 9.4	6.2 - 3.9	
12.	184 - 108	4.5 - 3.3	2.7 - 4.2	3.4 - 3.7	12.	120 - 106	4.3 - 3.6	2.4 - 3.9	4.3 - 6.0	
13.	53 - 48	7.5 - 6.7	4.3 - 4.4	9.5 - 8.5	13.	540 - 75	11.6 - 1.9	3.6 - 2.6	4.9 - 2.2	
14.	150 - 140	10.6 - 12.6	4.6 - 5.7	14.1 - 16.7	14.	136 - 93	7.8 - 7.3	4.5 - 6.2	9.7 - 10.4	
15.	181 - 111	10.2 - 10.6	8.2 - 5.5	13.0 - 12.9	15.	144 - 134	19.5 - 13.5	6.8 - 6.7	23.2 - 17.1	
16.	89 - 174	6.3 - 15.5	.9 - 2.9	6.3 - 16.9	16.	47 - 58	5.8 - 3.9	5.3 - 4.9	9.1 - 6.8	
17.	60 - 46	11.1 - 8.1	5.5 - 4.7	15.0 - 11.7	17.	41 - 134	.9 - 5.7	.7 - 5.5	.9 - 7.2	
18.	414 - 129	17.5 - 15.2	3.7 - 3.2	15.9 - 17.0	18.	178 - 175	4.7 - 2.7	8.6 - 2.6	8.3 - 2.4	
19.	144 - 146	2.9 - 1.2	2.8 - 3.1	3.2 - .8						
20.	216 - 181	.8 - 1.1	4.6 - 4.6	2.3 - 2.7						
21.	154 - 105	41.0 - 29.7	5.9 - 5.7	42.3 - 32.0						
22.	270 - 310	40.0 - 45.8	6.5 - 7.6	39.5 - 45.0						
Avg	130 - 114	9.4 - 9.6	3.8 - 3.9	10.8 - 10.6	Avg	129 - 105	7.2 - 5.3	4.0 - 4.0	8.6 - 6.8	

* These received 1 mg serotonin base, the remainder 2 mg.

† First figure of each column denotes values obtained at end of infusion; second figure indicates values of the combined subsequent two hourly specimens.

the close of the infusion, then fell to 5.3 in the 2-hour subsequent period. The urine volumes and the rates of excretion of sodium, potassium and chloride as affected by the infusion are shown in Table I. Whereas the urine sodium excretion rate diminished in 10 of the 22 control subjects following the infusion, it decreased in 15 of the 18 patients after the infusion with serotonin. In general, the chloride excretion rate was similar to that of sodium. No consistent trends were noted in either water excretion or urine potassium values.

The concentrations of electrolytes can be calculated readily from these data. The urine sodium concentration rose in 15 of the 22 control subjects after the infusion, but decreased in 15 of the 18 patients after serotonin. The average urine sodium concentration of the control group was 82 mEq/L. in the specimens obtained at the end of the infusion, and rose to 90 mEq/L. in those collected during the subsequent 2-hour period. In contrast, the average values of the serotonin group fell from 74 to 56 mEq/L.

These differences, employing averages as well as geometric means, were determined to be statistically significant ($p = <0.02$).

Discussion. These data indicate that the administration of small doses of serotonin creatinine sulfate may be associated with decreases in renal sodium excretion, occurring principally after an hour's infusion with this chemical. The conditions of the study were such that the changes noted could not be explained by variation in activity, diet or in the adequacy of urine collections as these were comparable for both groups. The mechanism, significance, and the effects of high dosage and prolonged administration remain to be determined. These observations support the view that electrolyte changes following rauwolfia administration may be related to serotonin activity. They also raise the question that serotonin may play a role in the physiologic regulation of sodium metabolism.

Summary. The administration of 1-2 mg of serotonin creatinine sulfate may be associated with significant sodium retention. Electrolyte changes following rauwolfia ad-

ministration may be related to this activity of serotonin.

1. Brodie, B. B., Pletscher, A., Shore, P. A., *Science*, 1955, v122, 968.
2. Perera, G. A., *J.A.M.A.*, 1955, v159, 439.

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Separation of Antibodies in Syphilitic Rabbit Sera by Electrophoresis-Convection.* (22312)

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The method of electrophoresis-convection has been used by Cann *et al.*(1-3) to obtain separations of a number of different types of antibodies. Their work suggested that it might be possible to separate the Wassermann reagin antibody from the antibody producing immobilization of spirochetes in the *Treponema pallidum* immobilization (TPI) test(4) and to relate these antibodies to electrophoretic serum components.

In this study a pool of syphilitic rabbit sera was fractionated by electrophoresis-convection and the resulting materials tested for the Wassermann reagin antibody by the VDRL flocculation test(5), tested for immobilizing antibodies in the TPI test and also tested for agglutinating activity(6). The fractions obtained were characterized by electrophoretic analysis.

Methods. The electrophoresis-convection apparatus used was that described by Raymond(7)‡. The operation of the apparatus was carried out in a coldroom at ca. 4°C. A 4 l bottle filled with buffer was placed in the buffer circulating system to increase the volume of circulating buffer to ca. 8 l. The increased buffer volume seemed desirable to minimize pH changes in the buffer during the course of the run. Protein concentrations were estimated by semimicro Kjeldahl nitrogen determinations and the factor 6.25 was used to convert nitrogen to protein. The relative composition of the serum fractions was determined by paper electrophoresis using the method developed by Grassmann and Hannig(8)§. The mobilities were determined by moving boundary electrophoresis using the Perkin-Elmer Model 38 Electrophoresis Apparatus. The determinations were carried out at a protein concentration of 0.3% in barbital buffer (pH 8.6, $\mu = 0.1$). The samples were simultaneously dialyzed for 48 to 72 hours against the same 2 l volume of barbital buffer. The specific conductance of the buffer after dialysis was used in the mobility calculations. The mobilities reported are the average of those calculated from photographs of the descending boundaries taken by the Longsworth scanning procedure, at 1, 1.5 and 2.0 hours. The VDRL slide flocculation test was used to measure the relative Wassermann reagin antibody content of the fractions. The serum fractions were not heat inactivated prior to testing. The TPI test with modifications(6) was carried out on serum fractions that had been diluted with normal rabbit serum (Pool I) to a final concentration of 2.0% of serum fraction protein and then heat inactivated at 56°C for 30 minutes. The samples tested were of the order of half serum

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‡ Manufactured by E. C. Apparatus Co., New York City.

§ The paper electrophoresis apparatus and densitometer were obtained from E. Miltenberg Inc., New York City.

TABLE I. Serologic Analysis.

	TPI		Agglutination		VDRL			
	Protein conc., %	Titer*	Protein conc., %	Titer‡	Protein conc., %	Undiluted	1:2	1:4
Pool VI (syphilitic) undiluted	6.8	1: 940	6.77	1: 80	6.8	2+	1+	neg
Pool VI 2%	2.0	1: 340	2.0	1: 20	2.0	±	neg	"
pH 7.5 Top	2.0§	1: 820	2.0§	1: 80	5.9	4+	3+	"
7.0 "	2.0§	1: 1170	2.0§	1:160	2.8[5]	2+	neg	"
6.5 "	2.0§	1: 1300	2.0§	1: 80	3.4	±	"	"
6.0 "	2.0§	1:10000	2.0§	"	3.4	±	"	"
5.5 "	1.0§	1: 1920†	1.0§	" †	1.8[5]	neg	"	"
Pool I (normal) undiluted	6.2	neg‖	6.22	undil. 1+	6.2	"	"	"

* Dilution of serum protein to produce 50% immobilization of spirochetes.
† Titer on basis of a 2.0% solution.
‡ Dilution of serum protein to produce a 2-3 agglutination of spirochetes.
§ Serum fraction protein concentration.
‖ Negative.

fraction protein and half normal rabbit serum protein. All the TPI titers were obtained from a single assay of high sensitivity and are reported as the dilution of the sample necessary to produce 50% specific immobilization of the spirochetes. The same samples prepared for the TPI assay were tested for *T. pallidum* agglutination activity by the method of McLeod and Magnuson(6). The titers reported for comparison were obtained from a single test and are the greatest dilutions of the serum fractions that produced 2 to 3+ spirochete agglutination.

Experimental. The syphilitic serum used in this study was an aliquot of a serum pool from 105 rabbits that had had syphilis for 4½ to 7½ months. They had been inoculated intratesticularly with the Nichols pathogenic strain of *T. pallidum*. The pooled serum and the serum fractions were refrigerated at −10 to −20°C except when fractionated or tested. A total volume of 135 ml of the syphilitic rabbit serum was processed in three batches of 45 ml each. The serum was diluted with an equal volume of buffer which gave a final protein concentration of 3.4%. Fresh sodium phosphate buffer was used for each run. The sample was dialyzed against the buffer in which it was to be fractionated. During each fractionation a current of 0.1 ampere and 8 to 10 volts (field strength ca. 2 volts cm^{-1}) was passed through the system for 94 to 96 hours. Fractionations were carried out in sodium phosphate buffers of 0.1 ionic strength and at a series of pH's 7.5, 7.0, 6.5, 6.0, and 5.5. The first run was made at pH 7.5 and the "bottom" from this run was then fractionated at pH 7.0. The process was repeated through the 5 different buffers. The corresponding portions from the fractionation of each of the 45 ml aliquots were pooled before proceeding to the fractionation at the next pH. At the end of this procedure there were 5 "top" fractions and one "bottom" fraction.

Results. The "bottom," which consisted principally of albumin with a little globulin, gave negative TPI, agglutination and VDRL tests and was discarded. The relative com-

TABLE II. Electrophoretic Analysis.

	Relative composition, %*				Mobilities (cm^2 $volts^{-1}$ sec^{-1})† × 10^5	
	Alb.‡	α-glob.	β-glob.	γ-glob.	β-glob.	γ-glob.
Pool VI	60 (67)	9 (11)	14 (10)	17 (12)		
pH 7.5 top	2	0	4	94		−1.2[8]
7.0 "	0	0	11	89		−1.4[2]
6.5 "	0	0	10	90	−3.5[2]	−1.6[3]
6.0 "	4	0	20	76	−3.6	−1.7[5]
5.5 "	8	6	30	56	−3.5[3]	−1.9

* Relative protein concentrations were estimated by paper electrophoresis except those values for Pool VI in parenthesis which were estimated by moving boundary electrophoresis (protein 1.7%, barbital buffer pH 8.6, $\mu = 0.1$).
† Mobilities were determined by moving boundary electrophoresis (protein 0.3%, barbital buffer pH 8.6, $\mu = 0.1$).
‡ Alb. = albumin; glob. = globulin.

position of the proteins in the 5 "top" fractions was determined by paper electrophoresis. It was found that they contained from 20 to 40% albumin. This indicated a poorer separation than had been previously reported for a similar electrophoresis-convection fractionation of rabbit serum(3). Serologic tests of the fractions showed little if any separation of antibody activities. Therefore each of the 5 "tops" was refractionated in the electrophoresis-convection apparatus using a buffer of the same composition as that in which it was originally separated. The 5 "bottom" fractions were discarded and the "top" fractions were concentrated by pervaporation, dialyzed against pH 7.0 phosphate buffer and the serologic tests carried out. In each of the tests a sample of the unfractionated serum, designated Pool VI, was run at the same protein concentration as the fractions being tested. The serologic results are shown in Table I. The relative composition of the serum fractions was determined by paper electrophoresis and the mobilities determined by moving boundary electrophoresis. The results of the electrophoretic analysis are presented in Table II.

Discussion. The electrophoretic results are in accord with those obtained previously by a similar fractionation of rabbit serum(3). There is a progressive increase in the mobility of the γ-globulin isolated at successively lower pH's and there is no detectable difference in the mobilities of the β-globulins obtained.

The VDRL test results indicate a relative concentration of the Wassermann reagin antibody in the fractions isolated at pH 7.5 and 7.0 and it is probable therefore that this antibody is associated with the slow γ-globulin. There is a relative concentration of the *T. pallidum* immobilizing activity in the fraction isolated at pH 6.0. The difference in titer between the pH 6.0 fraction and the fractions next higher in immobilizing activity corresponds to 2½ to 3 tubes of 2-fold dilution and is believed to be significant. The most reasonable interpretation is to ascribe the immobilizing activity to components in the fast γ-globulin. The serum protein fractions were obtained by a method that produces separations on the basis of differences in electrophoretic mobility. The possibility exists that the differences in antibody activity measured were due to a fractionation of the α or the β globulin components. There was no evidence obtained for differences in mobilities among the α components or the β components of the various fractions. The more likely interpretation however is to associate the differences in antibody activities that have been demonstrated with the differences in mobilities of the γ-globulin fractions that have been measured.

There does not appear to be any significant concentration of agglutinating activity in any of the fractions. This may be a reflection of the importance of more than one antibody in the agglutination reaction(9).

Summary. An electrophoresis-convection fractionation of a pool of syphilitic rabbit serum has been carried out. Significant differences in the mobilities of the γ-globulin components of these fractions have been demonstrated. No such differences in the mobilities of the β-globulin components were found. TPI, VDRL and agglutination studies were carried out. A comparison of the serologic and electrophoretic data indicates that the Wassermann reagin antibody is concentrated in the slow γ-globulin component and the *T. pallidum* immobilizing antibody is concentrated in the fast γ-globulin component. There is no evidence for the concentration of *T. pallidum* agglutinating activity in any of the fractions.

1. Cann, J. R., Brown, R. A., Gajdusek, D. C., Kirkwood, J. G., and Sturgeon, P., *J. Immunol.*, 1951, v66, 137.

2. Cann, J. R., Brown, R. A., Kirkwood, J. G., and Hink, J. H., *J. Biol. Chem.*, 1950, v185, 663.

3. Cann, J. R., Campbell, D. H., Brown, R. A., and Kirkwood, J. G., *J. Am. Chem. Soc.*, 1951, v73, 4611.

4. Nelson, R. A., Jr., and Mayer, M. M., *J. Exp. Med.*, 1949, v89, 369.

5. Harris, A., Rosenberg, A. A., and Del Vecchio, E. R., *J. Ven. Dis. Inform.*, 1948, v29, 72.

6. McLeod, C. P., and Magnuson, H. J., *Pub. Health Rep.*, 1953, v68, 747.

7. Raymond, S., PROC. SOC. EXP. BIOL. AND MED., 1952, v81, 278.
8. Grassmann, W., and Hannig, K., *Hoppe-Seyler's Z. physiol. Chem.*, 1952, v290, 1.
9. McLeod, C. P., and Stokes, P. S., *Pub. Health Rep.*, 1955, v70, 379.

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Serological Response to Japanese B Encephalitis Vaccine of Children and Horses Immune to St. Louis Virus.* (22313)

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In the spring of 1946 an opportunity was afforded one of us (W. McD. H.) to make serological tests on adult Japanese and American troops given a single injection (1 ml) of Japanese B encephalitis (JBE) mouse brain vaccine, then in current use in American troops. Japanese with elevated neutralizing antibody titers to JBE virus and without detectable complement fixing (C.F.) antibody prior to vaccination responded with elevated C.F. titers within 10 days after injection of a single dose of vaccine(1). This observation was subsequently confirmed by Sabin, Ginder, Matumoto and Schlesinger(2). On the other hand, American troops usually had neither neutralizing nor C.F. antibody responses after such an interval and only about 50% responded with significant neutralizing antibodies after the regular course of 3 injections; also, with rare exceptions, there was no C.F. antibody detectable at any time(1,3). This led us to wonder whether a rapid C.F. antibody response after an injection of JBE vaccine might be a specific means of determining whether an individual found to have JBE neutralizing antibody had been previously infected with JBE virus or whether such antibodies might be the result of an infection with St. Louis encephalitis (SLE), West Nile (WN) or another virus in this closely related group. In this connection, 2 of us (W. McD. H. and W. C. R.), while at the Hooper Foundation, had repeatedly observed in performing sero-diagnostic work for the arthropod-borne viral encephalitides that many humans and horses with SLE virus neutralizing and C.F. antibodies also had elevated serological titers to JBE virus. In some instances the titers to the latter agent were higher than those to SLE virus. Similar diagnostic rises occurred with both viruses. Obviously, if both viruses should be present in the same area this would pose a serious diagnostic problem. Also, as has already occurred in our experience and in that of others, antibodies for several of these viruses have been found in populations of other countries prior to isolation of any virus. Which virus gave rise to the antibodies has not always been obvious. The experiments reported in this paper were undertaken to see whether administration of JBE vaccine would permit of specific serologic differentiation for JBE virus infection.

Materials and methods. All sera after collection were frozen in rubber stoppered Pyrex glass tubes until tested. They were shipped with dry ice, then held at –25°C. All sera in a series from the same host and pertaining to one experiment were tested simultaneously. Neutralization tests were performed both by the intracerebral route (in 3 to 4-week-old mice) and by the intraperitoneal route (in

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TABLE I. Serological Response of Human Encephalitis Cases from Kern County, Calif., during Disease and Convalescence and Finally before and after an Injection of Japanese B Vaccine.

Name	Time relation to onset or vaccination (days)	St. Louis Neutralization Intracer.	St. Louis Neutralization Intraper.	St. Louis CF	Japanese B Neutralization Intracer.	Japanese B Neutralization Intraper.	Japanese B CF
		× 1000					
Goodloe	8 P.O.†	(1)* 39	—	(1)<4	(1) 100	—	—
		(2)* 120		(2) "			
	20 P.O.	(2) 220	—	(1) 64	(1) 100	—	—
				(2) "			
	27 P.O.	(1) 285	(1) >100	(1) "	(1) 1,600	—	—
				(2) "			
	53 P.O.	(2) 770	(1) 1,000	(2) "	(1) 1,000	—	—
	0 P.V.†	(1) 3,200	(1) 160	(1) 4	(1)<100	(1) 80	(1)<4
	11 P.V.	(1)10,000	(1) >320	(1) 32	(1) 1,000	(1)10,000	(1) 8
Leflore	16 P.O.	(1) 32	—	(1) 32	(1) 75	—	—
	42 P.O.	(1) 2,000	>1,000	(1)128	(1) 1,000	—	—
	140 P.O.	—	—	—	(1) 100	—	—
	0 P.V.	>100	>10	(1) 8	(1) 630	(1) 3,200	(1)<4
	11 P.V.	1,000	>320	(1) 8	(1) 320	(1) 6,300	(1) 8
Bugni	Onset	(1) 155	(1) 10	(1)<4	(1) 150	—	—
	45 P.O.	(1) 1,900	—	(1) 64	(1) 3,200	—	—
	120 P.O.	—	(1) 1,000	—	(1) 320	—	—
	155 P.O.	—	(1) 1,000	—	(1) 1,000	—	—
	0 P.V.	(1) 1,000	(1) >320	(1) 16	320	800	(1) 4
	11 P.V.	(1) 5,000	(1) >320	(1) 32	1,300	32,000	(1) 32

* No. in parentheses represent the date on which tests were performed. In any one section (post onset, or pre or post vaccination) in the same vertical column, all those tests with the same numeral in parentheses were performed at one time.

† P.O. = Post onset; P.V. = Post vaccination.

suckling mice) by the virus dilution method, using undiluted and unheated serum. The virus strains used were: for the intracerebral test, SLE, Webster strain, and JBE, Okinawa strain; and for the intraperitoneal test, SLE, Hubbard strain, and JBE, Nakayama strain. Incubation of serum and virus was for 2 hours at 37°C. All mice were of the CFW strain. Complement fixation tests were done by the method of Casals(4), employing serial dilutions of serum, 2 exact units of complement and incubation overnight at 4°C. The antigens were of the benzene-extracted type, using the method of España and Hammon(5). JBE chick embryo type of formalin inactivated vaccine of suitable potency was obtained from Doctor J. E. Smadel of the Army Medical Service Graduate School. Through the courtesy of Doctor W. A. Longshore of the California State Department of Public Health, 3 children whom we had diagnosed by serological methods in 1949 as cases of St. Louis encephalitis were bled on March 8, 1950, then given 1 ml of vaccine and bled 11 days later. Sera for retest taken during the acute and convalescent phase of their illnesses were still present in our deep freeze. Fourteen horses resident in Kern County were selected on the basis of earlier serological testing and antibody status. Two control horses were selected from a non-endemic area on the basis of an absence of detectable antibodies. Through the kindness of Doctor Ben H. Dean of the California State Department of Public Health the horses were bled, injected with 5 ml of vaccine each, then bled again 10 and 30 days later. Aliquots of serum samples from these horses were tested in 3 different laboratories, California State Department of Public Health (EHL), Hooper Foundation, U. of California (WCR) and University of Pittsburgh (WMH and GES), by a variety of methods. The results in the 3 laboratories were essentially alike, and those reported here are the results of tests performed at the University of Pittsburgh.

Results. 1. *Convalescent Patients.* The results of tests made during illness and convalescence, followed by those just before and after vaccination of the 3 children, are pre-

sented in Table I. It will be observed that just before vaccination all had detectable neutralizing and C.F. antibody to SLE virus. Goodloe had essentially no antibody of either type to JBE virus. Leflore had only neutralizing antibody, while Bugni had a detectable level of both types of antibody. Following vaccination all but Leflore showed an increase in JBE neutralizing antibody and all showed significant rises in JBE C.F. antibody. In

TABLE II. Serological Studies on Japanese B and St. Louis Antibody Response of California Horses Vaccinated with Japanese B Vaccine.

	Neutralization tests				C.F. tests	
	St. Louis		Japanese B		St.	
Horse	Intracer.	Intraper.	Intracer.	Intraper.	Louis	Jap B
Red	* 4,000	>50,000	20	1,800	<4§	<4
	† 800	"	130	160,000	32	32
	‡				16	16
Boots	4,000	"	1,000	3,000	<4	<4
	8,000	"	10,000	4,000	8	8
					4	<4
Pat	1,000	320,000	12	<100	<4	<4
	500	6,000,000	100	16,000	8	8
					4	4
King	1,000	500,000	250	6,000	<4	<4
	4,000	6,000,000	16,000	1,000,000	8	4
					<4	<4
Jackass	800	>1,000,000	200	<100	8	<4
	1,600	"	400	84,000	16	16
Black	6,300	"	700	200,000	<4	<4
	6,300	"	700	8,400,000	32	32
Marge	1,600	"	500	500	<4	<4
	16,000	"	2,000	>6,300	8	8
					4	<4
Sally	1,000	200,000	25	250	<4	<4
	5,000	400,000	400	250,000	64	64
					16	16
Boots	800	700,000	40	160	<4	<4
	500	1,600,000	25,000	1,000,000	16	16
					8	8
Apache	5,000	1,000,000	70	100	<4	<4
	1,000	>1,000,000	700	6,000,000	16	16
					8	8
Chief	6,000	>50,000	180	1,300	<4	<4
	6,300	"	4,000	1,000,000	16	16
					<4	8
Thunder	4,000	"	40	50	<4	<4
	4,000	"	800	100,000	32	32
					8	8
Pinto	500	>4,000,000	160	6,300	<4	<4
	2,500	"	16,000	1,000,000	16	16
					8	8
Easter	1	<600	16	<100	<4	<4
	50	"	16	"	8	8
					<4	<4
#1564 Cutter	1	<30	1	<130	<4	<4
	1	"	8	"	<4	<4
					<4	<4
#1556 Cutter	1	100	1	<130	<4	<4
	1	<32	8	"	<4	<4
					<4	<4

* Pre-inoculation bleedings. † 10-day bleeding. ‡ 30-day bleeding. § Reciprocal of dilution giving 2+ or higher complement fixation.

general, rises also occurred in SLE antibodies. However, end-points were not always attained, but in some where titrations were complete, significant rises were not demonstrated.

2. *Horses.* Results of the tests on the horses are presented in Table II. The prevaccination sera of 13 of the 16 horses contained significant neutralizing antibody to SLE virus and most of these 13 also had detectable JBE neutralizing antibody titers. Easter had none detectable by the intracerebral method but the intraperitoneal test was not performed with a small enough amount of virus to eliminate the possibility of a trace of antibody. The last 2 horses (controls) in the Table can be considered negative for neutralizing and C.F. antibodies.

Following vaccination, C.F. antibody titers rose rapidly to both SLE and JBE in all those with previous SLE and/or JBE neutralizing antibody. Higher titers were found after 10 days than after 30. Easter gave this same type of response. However, those unequivocally negative for SLE neutralizing antibody prior to vaccination showed no response. Neutralizing antibody response to JBE in the 10-day interval (not tested after 30 days) also appears to have been very pronounced in most horses originally immune to SLE, and the rise was more conspicuous than that to SLE. Easter and the two with no previous immunity showed no detectable response.

Discussion. Both children and horses (resident in the Western United States) and naturally immune to what we assume must be St. Louis virus responded serologically to a single injection of a killed-virus JBE vaccine. This response was indistinguishable from that of persons immune to JBE. The C.F. antibody response was most dramatic, since nonimmunes fail to develop any antibody of this type, regardless of time or even after repeated injections. If one assumes that the horses and children had not been previously infected with JBE virus, the secondary type response demonstrated by JBE vaccine is not specific for previous JBE infection, and the test does not serve any useful purpose in differentiating between those two viruses. It is, however, in all probability a specific secondary immunologic response to a common antigen present in both JBE and SLE viruses.

It will be recalled, however, that in addition to the rapid rise in C.F. antibody a very striking and rapid rise generally occurred also in JBE neutralizing antibodies. In view of the low response rate (7 to 30%) to even 3 injections of vaccine of this same type then in use in normal American troops(6), one of the factors leading to its discontinuance in military preventive medicine, the response in St. Louis immunes, assumes particular interest. The following questions are raised: (1) Is it possible that the few American adults (troops) who had good serological responses to JBE, *i.e.*, high neutralizing antibody titers and positive C.F. tests, represented those few individuals with immunity to SLE, and (2) could this heterologous, booster phenomenon be used for a practical purpose in immunization?

Since JBE immunization with currently available killed vaccine has not been used recently in military groups because its effectiveness has been questioned, it is possible that another antigenically related virus of a more benign nature might be employed as an infecting agent to give basic primary group immunity. This then could be supplemented with an injection of killed JBE vaccine to obtain the response noted in these experiments. Certain strains of West Nile virus have already been demonstrated to be quite benign(7) and suggest themselves for this purpose.

With this in view, experimental work in laboratory animals has been undertaken by the University of Pittsburgh group and will be the subject of subsequent reports(8).

Summary and conclusions. Three children convalescent from St. Louis encephalitis and a group of 16 horses, 13 of which had naturally acquired antibodies to St. Louis virus, were given one injection with a killed Japanese B encephalitis virus vaccine. After 10 or 11 days all individuals previously infected with St. Louis virus showed complement fixing antibody rises to Japanese B and St. Louis viruses and most also showed neutralizing

antibody rises, particularly to Japanese virus. Those horses not previously immune to St. Louis virus showed no response. The response of these St. Louis immune hosts was identical to that shown by persons with naturally acquired immunity to Japanese virus. Normal individuals show no such response even after several injections of vaccine. This immediate, secondary type immunologic response to an injection of vaccine is not specific for Japanese virus but to an antigen common to Japanese and St. Louis viruses and probably to other viruses in this complex. It is suggested that immunity acquired by active infection with one member of the group may serve as good basic or primary immunization for other representatives and a high and probably effective protection response can be stimulated subsequently by giving one "booster" injection of a killed virus vaccine representing an immunologically related virus.

1. Hammon, W. McD., unpublished.

2. Sabin, A. B., Ginder, D. R., Matumoto, M., and Schlesinger, R. W., PROC. SOC. EXP. BIOL. AND MED., 1947, v65, 135.

3. Ginder, D. R., Matumoto, M., Schlesinger, R. W., and Sabin, A. B., PROC. SOC. EXP. BIOL. AND MED., 1947, v65, 130.

4. Casals, J., *J. Immunol.*, 1947, v56, 337.

5. España, C., and Hammon, W. McD., *ibid.*, 1948, v59, 31.

6. 406 Medical General Laboratory, Ann. Historical Rep., 1952, Professional Sect. p43.

7. Southam, C. M., and Moore, Alice E., *J. Immunol.*, 1954, v72, 446.

8. Hammon, W. McD., and Sather, G. E., PROC. SOC. EXP. BIOL. AND MED., 1956, v91, 521.

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Immunity of Hamsters to West Nile and Murray Valley Viruses Following Immunization with St. Louis and Japanese B.* (22314)

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In a previous paper(1) it was shown that human convalescents of St. Louis encephalitis (SLE) virus and horses with naturally acquired SLE immunity responded quickly with a secondary type of serological response to Japanese B (JBE) virus after one injection of JBE virus, killed vaccine. This paper deals with animal challenge by the subcutaneous route with West Nile (WN) and Murray Valley encephalitis (MVE) viruses following immunization with JBE and SLE viruses belonging to the same immunologic group. Serological crossing between SLE, JBE, WN, and MVE viruses has been demonstrated by neutralization, complement fixation, and hemagglutination tests by a large number of workers and antigenic overlapping of this group is well recognized. Cross-protection tests using the intracerebral challenge of immunized mice have not shown such crossing or have given inconclusive results with two exceptions as noted below. On the basis of the usual lack of cross-protection in mice has rested the principal method of differentiation of members of this group as distinct viruses. Recently, however, Ruchman reported that he had demonstrated cross-protection between SLE and WN using immune rabbits and guinea pigs as test animals(2). His criterion of demonstrated disease in those animals involving tests with recognized strains of SLE and WN was the development of fever. Cross vaccination studies by Pond, Russ, Rogers, and Smadel(3) have demonstrated the relationship between JBE and MVE, since JBE vaccinated mice were satisfactorily protected against peripheral challenge with MVE virus.

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MVE vaccinated mice, however, were not significantly protected against JBE, although there was a suggestion of slight crossing.

In our work, Syrian hamsters have been found susceptible to high dilutions of MVE virus by peripheral routes of infection. Previously, Smadel and others had shown them to be highly susceptible to WN by these routes(4). Because of the low degree of susceptibility of mice by peripheral routes, we felt that hamsters would be a more suitable laboratory animal for demonstrating cross-resistance between certain viruses of this group, if such existed.

Materials and methods. Viruses used were JBE, Nakayama strain, mouse passage 44; SLE, Webster strain, mouse passage number unknown; MVE, Strain #1, mouse passage 12; and WN, B956, mouse passage 28. Stock virus was prepared as 20% infected mouse brain suspension in 50% normal rabbit serum (inactivated at 56°C for 20 minutes) broth and stored in sealed glass ampoules in a CO_2 ice chest. Infectivity of the virus was determined by intracerebral inoculation of 3-week-old CFW mice with 0.03 ml of serial 10-fold virus dilutions made in 0.2% bovine albumen broth. Hamsters were obtained from the Lakeview Hamster Colony of New Jersey. Hamsters from this breed had been found susceptible to infection with this group of viruses. Animals previously tested from a local breeder had proved resistant to SLE virus by even the intracerebral route. Groups of hamsters were immunized when 6 weeks of age to either JBE or SLE viruses by a series of 3 intraperitoneal injections of live virus given as follows: *JBE*: first injection, 0.1 ml 10^{-2} dilution of virus; 10 days later, 0.1 ml 10^{-1} dilution of virus; 10 days later, 0.2 ml 10^{-1} dilution of virus. Challenge was made 12 days later. *SLE*: first injection, 0.1 ml 10^{-2} dilution of virus; 12 days later, 0.1 ml 10^{-1} dilution of virus; 7 days later, 0.2 ml 10^{-1} dilution of virus. Challenge was made 13 days later. For challenge controls, normal hamsters of the same shipment were set aside at the beginning of the immunization period. The neutralization index of serum was determined by employing serial dilutions of virus mixed with undiluted serum, incubated at 37°C for 2 hours, and inoculated by the intracerebral route into 3-4-week-old mice.

TABLE I. Susceptibility of Hamsters by Route of Inoculation.*

Virus	Log LD_{50} of endpoint dilution		
	Intracer.	Intraper.	Subcut.
Japanese B	7.0	<1.0	—
St. Louis	7.8	<1.0	—
Murray Valley	8.0	8.1	7.0
West Nile	9.5	9.2	8.7

* Inoculum—.1 ml.

Results. 1. The susceptibility of hamsters to these viruses as determined by route of inoculation (Table I). The inoculum was 0.1 ml. Both MVE and WN viruses were shown to be highly pathogenic by the peripheral routes tested, while neither JBE nor SLE exhibited this peripheral pathogenicity. The four viruses, JBE, SLE, MVE, and WN were all pathogenic by the intracerebral route.

2. Immunization and Cross Challenge. Two groups of hamsters were immunized, one to JBE virus and the other to SLE by the methods described. The results of homologous and cross challenge tests are shown in Table II. Since neither JBE nor SLE were pathogenic by a peripheral route, it was necessary to use the intracerebral route of inoculation for challenge with the homologous viruses. For both MVE and WN viruses (heterologous challenge) the *subcutaneous* route of inoculation was used. It will be observed that animals immunized with JBE virus were solidly immune not only to the homologous virus (JBE) but also to both the MVE and WN viruses (protection index greater than 5 to 7 logs). This experiment was repeated with similar results. Immunization with SLE also resulted in complete protection against challenge with the homologous (SLE) virus, but it may be observed that SLE immunization did not result in complete protection against either MVE or WN viruses but that significant protection, nevertheless, did occur. In the case of both MVE and WN viruses protection was most obvious against the most concentrated virus inoculum. This phenomenon frequently observed after ho-

TABLE II. Results of Virus Challenge of Immunized Hamsters.

Immunizing virus	Challenge virus	Route of challenge	Virus dilution 10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}	Log LD_{50}	Log protection index
JBE	JBE	i.c.	0/4*	0/4	0/4	0/4							<1.0	>7.5
None	"	"					4/4	4/4	4/4	3/4	1/4		8.5	
JBE	MVE	s.c.	0/4	0/4	0/4	0/4	0/4	0/4					<1.0	>5.2
None	"	"					4/4	3/4	1/4	0/4			6.2	
JBE	WN	"	1/4	0/4	0/4	0/4	0/4	0/4					<1.0	>7.3
None	"	"						4/4	2/4	4/4	0/4	0/4	8.3	
SLE	SLE	i.c.	0/4	0/4	0/4	0/4	0/4						<1.0	>6.8
None	"	"					4/4	4/4	3/4	4/4	2/4		7.8	
SLE	MVE	s.c.	0/4	1/4	2/4	3/4	3/4	2/4	0/4				?	?
None	"	"				4/4	4/4	2/4	0/4	0/4			6.0	
SLE	WN	"	0/4	1/4	3/4	2/4	2/4	1/4	1/4				?	?
None	"	"					4/4	4/4	3/4	3/4	4/4	0/4	9.3	

* Mortality ratio = No. dying/No. inoculated.

mologous challenge of incompletely immunized animals has been discussed by Schlesinger(5). Because of this zone-like reaction the LD_{50} and the protection index were not calculated.

3. *Antibody Response as Related to Survival.* To determine whether resistance to challenge with any virus correlated with the neutralizing antibody titer for that virus, certain animals prepared for use in the above experiments were sacrificed and bled. This was planned after completion of the challenge of the SLE immunized hamsters, so in this instance 4 hamsters surviving homologous challenge were bled. Four JBE immune hamsters which had not been challenged were sacrificed and bled at the time all the others were challenged. Sera from these 4 animals were pooled. All these sera were tested for neutralizing antibody titer against MVE and WN viruses as well as against the homologous viruses. The results are shown in Table III. It will be noted that in the case of those animals immunized with SLE virus, even though bled after intracerebral challenge with homologous virus, thus receiving more immunization than those that were immunized with JBE virus, the antibody titer to MVE and WN viruses was much lower than in those animals immunized with JBE virus. In a parallel manner, the SLE immunized group were less resistant to challenge inoculation with the two heterologous viruses than were those immunized with JBE.

Discussion. The importance of demonstrating cross-protection amongst this group of viruses, especially by peripheral challenge, is evident when we realize that this route more nearly represents the type of natural exposure expected in an arthropod-borne disease. The possible practical application of such a finding was conceived as a result of earlier work on the antibody response to one injection of JBE virus vaccine of children and horses in California who had had a previous, naturally acquired SLE infection(1). Since it was not practical to challenge either the children or the horses with a peripheral inoculation of JBE to demonstrate whether they were really immune, as suggested by the antibody response, it was necessary to find a suitable small laboratory animal that was susceptible to peripheral challenge. The hamster has served such a purpose for challenge with MVE and WN viruses, and cross immunity to each of these has been shown following immunization with either JBE or SLE virus.

TABLE III. Neutralization Indices of Sera from Immunized Hamsters Used in Challenge Tests.

Immune hamster serum	Virus: Homologous JBE	Homologous SLE	Heterologous MVE	Heterologous WN
Japanese B (pool)	2000	—	214	11300
St. Louis				
Serum 1	—	514	<5	650
2	—	163	15	1180
3	—	<163	8	650
4	—	163	10	<206

The development of cross immunity in hamsters, together with the antibody findings with SLE immune children and horses injected with JBE vaccine lend further support to the hypothesis that any one virus in the group (possibly a benign strain of WN) if used as a living immunizing agent, might furnish basic, partial protection against all and could be effectively supplemented by a single injection of a specific killed vaccine for the virus found in the area under consideration. Repeated injections of killed vaccine alone, for JBE, the only member of this group of viruses used extensively for immunization, have been shown to be relatively ineffective in producing a neutralizing or complement fixing antibody response in adult man, and hence of questionable value in affording clinical protection. Further work along these lines, using hamsters and monkeys with other combinations of viruses in this group, is now in progress and will be reported shortly.

Summary and conclusions. An available strain of Syrian hamsters was found to be highly susceptible to WN and MVE viruses by peripheral routes of inoculation. Groups of animals of this species were then immunized with JBE and SLE viruses and subsequently challenged subcutaneously with serial dilutions of WN and MVE viruses. Complete protection against these two agents was afforded by JBE immunization, and protection of a lesser degree obtained by SLE immunization. Neutralizing antibody titers of the immunized hamsters to the 2 heterologous challenge viruses paralleled their peripheral resistance in that the JBE virus immunes had higher neutralizing antibody titers to WN and MVE viruses than did the SLE virus immunes. These findings together with previously reported data support the hypothesis that cross immunity to these viruses probably occurs in man and that there might be some practical application of this concept in the field of human immunization. Further work with animals using other virus combinations is in progress.

1. Hammon, W. McD., Sather, G. E., Lennette, E. H., and Reeves, W. C., PROC. SOC. EXP. BIOL. AND MED., 1956, v91, 517.

2. Ruchman, I., *Fed. Proc.*, 1953, v12, 456; personal communication.

3. Pond, W. L., Russ, Sudie B., Rogers, Nancy G., and Smadel, J. E., *J. Immunol.*, 1955, v75, 78.

4. Watson, D. W., and Smadel, J. E., PROC. SOC. EXP. BIOL. AND MED., 1943, v52, 101.

5. Schlesinger, R. Walter, *J. Exp. Med.*, 1949, v89, 507.

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